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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

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Volume LV.

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JULY TO DECEMBER, 1928

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BIOLOGICAL BULLETIN

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THE MARINE BIOLOGICAL LABORATORY.

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I. TRUSTEES.

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to serve until 1928.

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C. R. Stockard, Cornell University Medical College.

Executive Committee of the Board of Trustees.

Frank R. Lillie, *Ex. Off. Chairman.*
Merkel H. Jacobs, *Ex. Off.*
Lawrason Riggs, Jr., *Ex. Off.*
Otto C. Glaser, to serve until 1928.
Caswell Grave, to serve until 1928.
E. G. Conklin, to serve until 1929.
C. R. Stockard, to serve until 1929.

THE LIBRARY COMMITTEE.

C. E. McCLUNG, *Chairman.*
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II. ACT OF INCORPORATION.

No. 3170

COMMONWEALTH OF MASSACHUSETTS.

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardiner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand, Eight Hundred and Eighty-Eight.

[SEAL] HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY.

I. The annual meeting of the members shall be held on the second Tuesday in August, at the Laboratory, in Woods Hole, Mass., at 12 o'clock noon, in each year, and at such meeting the members shall choose by ballot a Treasurer and a Clerk, who shall be, *ex officio,* members of the Board of Trustees, and Trustees as hereinafter provided. At the annual meeting to be held in 1897, not more than twenty-four Trustees shall be chosen, who shall be divided into four classes, to serve one, two, three, and four years, respectively, and thereafter not more than eight Trustees shall be chosen annually for the term of four years. These officers shall hold their respective offices until others are chosen and qualified in their stead. The President of the Corporation, the Director and the Associate Director of the Laboratory, shall also be Trustees, *ex officio.*

II. Special meetings of the members may be called by the Trustees to be held in Boston or in Woods Hole at such time and place as may be designated.

III. The Clerk shall give notice of meetings of the members by publication in some daily newspaper published in Boston at least fifteen days before such meeting, and in case of a special meeting the notice shall state the purpose for which it is called.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. The Trustees shall have the control and management of the affairs of the Corporation; they shall present a report of its condition at every annual meeting; they shall elect one of their number President of the Corporation who shall also be Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

VI. Meetings of the Trustees shall be called by the President, or by any two Trustees, and the Secretary shall give notice thereof by written or printed notice sent to each Trustee by mail, postpaid. Seven Trustees shall constitute a quorum for the transaction of business. The Board of Trustees shall have power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient.

VII. The accounts of the Treasurer shall be audited annually by a certified public accountant.

VIII. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

IX. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

X. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

IV. THE REPORT OF THE TREASURER.

To the Trustees of the Marine Biological Laboratory.

Gentlemen: As Treasurer of the Marine Biological Laboratory, I herewith submit my report for the year 1927.

The books have been audited by Messrs. Seamens, Stetson & Tuttle. A copy of their report is on file at the laboratory and is open to inspection by any member of the Corporation.

There were no changes in the investments in the Endowment Fund and that Fund at the close of the year consisted of securities of the book value of $906,337.50 and cash of $112. The income from the Endowment Fund for the year was $47,583 and the fee of the Trust Company as Trustee was $787.50, leaving a net income from the Endowment Fund of $46,795.50. The full list of the securities will be found in the Auditors' report.

At the end of the year the Lucretia Crocker Fund consisted of securities of the book value of $3,590.59 and cash of $1,093.17.

During the year a fund of Two Thousand Dollars ($2,000) invested in a note secured by Chicago real estate was presented to the Laboratory to found the Ida H. Hyde Scholarship and the fund remained invested in this security at the end of the year.

The Retirement Fund consisted of Seven Thousand Dollars ($7,000) invested in participations in bonds secured by mortgages on New York City real estate and $147.76 in cash.

The land, buildings, library and equipment of the Laboratory including the new apartment house and dormitory but excluding the Gansett and Devils Lane property represents an investment of $1,545,616.36, and after deducting $136,181.18 for depreciation, a book value of $1,409,435.18.

During the year the following donations were received:

From General Education Board for improving the facilities of the Library	$15,000
From Dr. Frank R. Lillie for grading and planting	1,500

During the year Two Thousand Dollars ($2,000) was paid off on account of the Danchakoff mortgage, and the indebtedness of the Laboratory at the end of the year consisted of $6,542.83 in accounts payable, and $42,500 in mortgages on its real estate.

The expenditures closely approximated the estimates for the year and including an item of depreciation of almost $29,000 exceeded the income for the year by $91.06. Against this item of depreciation the sum of almost $20,000 was expended out of current funds upon permanent improvements and equipment.

Since January 1, 1916, the Laboratory has adopted the policy of charging income and crediting reserve for depreciation each year with 2 per cent. of the book value of the buildings and 5 per cent. of the book value of equipment and library. This depreciation at the end of the year 1927 amounted to $136,181.18. It is interesting to note, however, that against this the Laboratory has spent from current cash approximately $141,000 in permanent improvements, thus more than meeting the depreciation charge by improvements paid for out of income.

Following is the balance sheet at the end of the year and the condensed statement of income and outgo for the year, also the Surplus Account. The figures are those reported by the Auditors, arranged in the case of Exhibit B so as to conform to the system followed in previous reports.

EXHIBIT A.

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1927.

Assets.

Endowment Fund Assets:			
Securities in Hands of Trustee—Schedule I.		$ 906,337.50	
Investment Cash in Hands of Trustees		112.00	
		$ 906,449.50	
Lucretia Crocker Fund Assets,			
Securities—Schedule II.....	$ 3,590.59		
Cash—Schedule II.........	1,093.17	4,683.76	
Ida H. Hyde Fund Assets,			
Securities		$ 2,000.00	$ 913,133.26
Plant Assets:			
Land—Schedule III...........	$113,603.05		
Buildings—Schedule III........	966,279.78		
Equipment—Schedule III.......	126,197.40		
Library—Schedule III.........	90,682.87	$1,296,763.10	
Less Reserve for Depreciation		136,181.18	
		$1,160,581.92	
Cost of New Dormitory and Apartment House Buildings to December 31, 1927—Schedule IV		$ 248,853.26	
Cash in Dormitory Building Fund		3,590.31	$1,413,025.49
Current Assets:			
Cash,			
In New York Bank	$ 2,335.99		
In Hands of Trustee	2,200.00		
In Falmouth Bank	1,558.19		
Petty Cash	500.00	$ 6,594.18	
Accounts—Receivable		20,440.55	
Inventories,			
Supply Department	$ 30,802.33		
Biological Bulletin	6,237.30	37,039.63	
Investments,			
Devil's Lane Property.....	$ 33,395.51		
Gansett Property	1,769.35		
Stock in General Biological Supply House, Inc........	12,700.00		
Retirement Fund Assets ...	7,147.76	55,012.62	
Prepaid Insurance		4,378.33	123,465.31
			$2,449,624.06

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Liabilities.

Endowment Funds:			
Friendship Fund, Inc.		$ 405,000.00	
John D. Rockefeller, Jr.		400,000.00	
Carnegie Corporation		100,000.00	
Gain on sale of Securities		1,449.50	
		906,449.50	
Lucretia Crocker Fund		4,683.76	
Ida H. Hyde Fund		2,000.00	$ 913,133.26
Plant Funds:			
Rockefeller Foundation		500,000.00	
Friendship Fund Gift of 1925		221,608.61	
General Education Board for Buildings		250,000.00	
General Education Board for Books	$25,000.00		
Less Unexpended in Current Cash	1,300.50	23,699.50	
Other Investments in Plants from Gifts and from Current Funds		399,525.38	
		$1,394,833.49	
Mortgages on Drew and Danchakoff Estates		17,500.00	
Suspense—Interest on Building Fund Cash;		692.00	1,413,025.49
Current Liabilities and Surplus:			
Mortgage Note on Devil's Lane Property..		25,000.00	
Accounts—Payable		6,542.83	
Items in Suspense (net)		222.47	
		$ 31,765.30	
Current Surplus—Exhibit C		91,700.00	123,465.31
			$2,449,624.06

EXHIBIT B.

MARINE BIOLOGICAL LABORATORY, INCOME AND EXPENSE, FOR THE YEAR ENDED DECEMBER 31, 1927.

	Total.		Net.	
	Expense.	Income.	Expense.	Income.
Income, Endowment Fund		$ 47,583.00		$47,583.00
Donations (See Current Surplus)				
Instruction	7,829.30	10,640.00		2,810.70
Research	3,598.86	14,525.00		10,926.14
BIOLOGICAL BULLETIN and Membership Dues	7,117.54	7,659.51		541.97

Supply Department, Schedule IV	52,174.46	59,820.90		7,646.44
Mess, Schedule V	33,085.42	36,180.30		3,094.88
Dormitories, Schedule VI	25,870.11	12,865.29	13,004.82	
Interest and depreciation charged to above three departments, See Schedules IV, V and VI	*29,719.11*			29,719.11
Dividends on Stock, General Biological Supply House, Inc.		2,540.00		2,540.00
Rent of Danchakoff Cottages	449.99	750.00		300.01
Rent of Microscopes		370.00		370.00
Rent of Garage, Railway, etc.		356.91		356.91
Rent of Newman Cottage	164.08	150.00	14.08	
Interest on Bank Balances		137.20		137.20
Medical Fees		114.00		114.00
Sundry Items		15.31		15.31
Maintenance of Plant:				
New Laboratory Expense	15,044.43		15,044.43	
Maintenance of Buildings and Grounds	13,296.19		13,296.19	
Chemical and Special Apparatus Department	9,088.83		9,088.83	
Library Department Expenses	7,958.73		7,958.73	
Carpenter Department Expenses	1,123.04		1,123.04	
Truck Expenses	1,203.52		1,203.52	
Sundry Expenses	814.22		814.22	
Bar Neck Property Expenses	405.00		405.00	
Evening Lectures	159.51		159.51	
Workmen's Compensation Insurance	627.43		627.43	
General Expenses:				
Administration Expenses	12,335.00		12,335.00	
Interest on Loans	1,168.00		1,168.00	
Endowment Fund Trustee	787.50		787.50	
Bad Debts	230.20		230.20	
Contribution for Research Space, Naples Zoological Station	250.00		250.00	
Reserve for Depreciation	28,736.23		28,736.23	
Excess of Expense over Income carried to Current Surplus—Exhibit C		91.06		91.06
	$193,798.48	$193,798.48	$106,246.73	$106,246.73

EXHIBIT C.

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT, YEAR ENDED DECEMBER 31, 1927.

Balance, January 1, 1927		$ 83,503.64
Add:		
Donations Received,		
From General Education Board for purchase of Books for Library		15,000.00
From Dr. Frank R. Lillie for Grading, Planting, etc. on Laboratory Grounds and around Drew House, Apartment House, and Whitman House		1,500.00
Income of Retirement Fund Assets		222.82
Reserve for Depreciation charged to Plant Fund		28,736.23
		$128,962.69
Deduct:		
Payments from Current Funds during Year Plant Assets as shown in Schedules III and III-*a*,		
Cost of completing Sea Wall	$ 435.50	
Buildings	7,303.90	
Equipment	4,312.23	
Library Books, etc.	7,623.94	
New Dormitory and Apartment House	251.57	
	$19,927.14	
Payments from above Donations charged to Plant Assets General Education Board, Purchase of Books	13,744.48	
Dr. Frank R. Lillie, Grading, etc.	1,500.00	
Payment on Danchakoff Mortgage	2,000.00	
Balance of Income and Expense Account—Exhibit B	91.06	37,262.68
Balance, December 31, 1927—Exhibit A		91,700.01

Respectfully submitted,
LAWRASON RIGGS, JR.,
Treasurer.

V. THE REPORT OF THE LIBRARIAN, DECEMBER 31, 1927.

The expenditures of the Library during 1927 totalled $26,939.67; segregated under the following headings: books, $527.20; serials, $3,325.57; binding of current serials, etc., $997.25; supplies, $541.88; express, $253.53; salaries, $5,650.00; miscellaneous salaries, $1,550.50; and General Education Board Fund, appropriated for back sets, $14,093.74. The total appropriation was $27,400.00, apportioned as follows: books, $500.00; serials, $3,000; binding, $1,000; supplies, $500.00; express, $200.00; salaries, $5,650.00; miscellaneous salaries, $1,550.00; and General Education Board appropriation, $15,000. It will be noted that the greatest part of the unused total occurred under the fund for back sets. The sum of $906.26 was carried on into 1928 and was in fact expended before January 15th, so that the expenditures all along the line, show a slight over-running of the appropriation. The most interesting item in this respect is that for serials. A special point of this condition was made by the Librarian in the 1926 Annual Report and in the informal report given to the corporation last August. Either an increase in appropriations will have to be made for this purpose, or some of the current serials must be dropped. For 1928, an increase was granted by the Executive Committee and this will have to be further increased for 1929. It will be noticed that of the total $26,939.67, $19,739.17 was expended on material and acquisitions, and $7,200.50 on salaries to carry on the work of the Library.

The Library now contains 22,762 bound volumes, most of these coming under the category of serials and books, and in about the proportion of 8 to 1. Of these, 4,154 were acquired this year, 657 by binding current serials; 674 by binding back sets; and the others by new back sets and books. Besides these volumes, the reprints number 43,000. Only 5,000 of these were catalogued and filed during this year.

The Library receives 764 serials currently and in addition to these periodicals, subscribes to 36 books and monographs that are being issued serially, 800 serial publications in all. Of these 764 periodicals, 136 were new this year; and of the 36 books and monographs, 5 are new. The new periodicals for 1927 are not all by paid subscription, but 87 were acquired by exchange with the BIOLOGICAL BULLETIN (67 copies of the BIOLOGICAL BULLETIN being sent out); and 19 by gift, while only 30 new paid subscriptions have been added. The total number of paid subscriptions including the serially issued books is now 270; and the total number of exchange, 274; and the gifts, 256. We have purchased 180 new books, and have received from publishers, 112, and from authors, 24, and from other sources, 144; 460 in all. These gifts were as follows:

From the publisher, P. Blakiston's Son & Co., Craigie, E. Horne: *An Introduction to the Finer Anatomy of the Central Nervous System Based upon That of the Albino Rat*; Evans, C. Lovatt: *Recent Advances in Physiology*; Gould, George M.: *Medical Directory*; Hawk, Philip B.: *Practical Physiological Chemistry*; Lewis, F. T. and Bremer, J. L.: *A Text-book of Histology arranged upon an Embryological Basis*; Meyers, Milton K., Editor: *Lang's German-English Medical Dictionary*; Pryde, John: *Recent Advances in Biochemistry*; Stitt, E. R.: *Practical Bacteriology*; Youngken, Heber W.: *Pharmacognosy*.

Gebruder Borntraeger: Dürken, Bernhard: *Allgemeine Abstammungslehre*; Herter, Konrad: *Tastsinn, Strömungssinn und Temperatursinn der Tiere und die diesen Sinnen zugeordneten Reaktionen*; von Buddenbrock, W.: *Grundriss der vergleichenden Physiologie*.

Chicago University Press: Newman, H. H.: *Evolution, Genetics, and Eugenics*; Newman, H. H., et al: *Nature of the World and of Man*.

Columbia University Press: Chandler Chemical Laboratories: *Contemporary developments in Chemistry*.

Detroit Digestive Ferments Co.: *Manual of Dehydrated Culture Media and Reagents*.

E. P. Dutton Co.: Einstein, Albert: *The Theory of Brownian Movement*; Freundlich, Herbert: *New Conceptions in Colloidal Chemistry*; Nernst, W.: *The New Heat Theorem: its Foundations in Theory and Experiment*; Ostwald, Wolfgang: *Practical Colloid Chemistry*; Stock, Alfred: *Structure of Atoms*.

Friederichsen & Co.: Michaelson, W., Editor: *Beiträge zur*

Kenntnis der Land-und Süsswasserfauna Deutsch-Südwestafrikas; Michaelsen, W., Editor: *Beiträge zur Kenntnis der Meeresfauna Westafrikas.*

Ginn & Co.: Miller, Dayton C.: *Laboratory Physics.*

Harcourt, Brace & Co.: von Uexküll, J.: *Theoretical Biology.*

Hokuryukwan & Co. Ltd.: Hirase, S. et al: *Figuraro de Japanaj Bostoj.*

Alfred A. Knopf, Inc.: Pearl, Raymond: *Biology of Population Growth*; Perrier, Edmond: *The Earth before History, Man's Origin and the Origin of Life*; Wheless, Joseph: *Is it God's Word?*

Lea and Febiger: Berkeley, W. N.: *The Principles and Practice of Endocrine Medicine*; DuBois, E. F.: *Basal Metabolism in Health and Disease*; Wiggers, Carl J.: *Modern Aspects of the Circulation in Health and Disease.*

Lewis, H. K. & Co.: Boes, P. K.: *X-ray Apparatus; its Arrangement and Use.*

J. P. Lippincott Co.: Addison, W. H. F.: *Piersol's Normal Histology*; Craig, C. F.: *Manual of the Parasitic Protozoa of Man*; Meyer, H. H. and Gottlieb, R.: *Experimental Pharmacology.*

Longmans, Green & Co.: MacLeod, John J. R.: *Carbohydrate Metabolism and Insulin.*

McGraw-Hill Book Co.: Allen, E. S.: *Six-place Tables*; Bancroft, Wilder D.: *Applied Colloid Chemistry*; Fernald, H. T.: *Applied Entomology—an Introductory Text-book of Insects in their Relations to Man*; Pearse, A. S.: *Animal Ecology*; Rogers, Charles G.: *Textbook of Comparative Physiology*; Shull, Charles: *Heredity.*

The Macmillan Co.: Adams, L. A.: *Necturus*; *A Dissection Guide*; Baitsell, Geo. A.: *Manual of Biological Forms*; Bernard, Claude: *Introduction to the Study of Experimental Medicine*; Billroth, Theodor: *Medical Sciences in the German Universities*; Brinkley, Stuart R. and Kelsey, E. B.: *Laboratory Manual arranged to accompany "Principles of General Chemistry"*; Cahn, Alvin R.: *The spiny dogfish*; *A Laboratory Guide*; Creaser, C. W.: *The Skate*; *A Laboratory Manual*; Frazer, James G.: *The Worship of Nature,* vol. I; Jeffrey, Edw. C.: *Coal and Civi-*

lization; Kerr, J. Graham: *Evolution*; Needham, Joseph: *Science, Religion, and Reality*; Newman, Horatio H.: *The Gist of Evolution*; Smuts, J. C.: *Holism and Evolution*; Woodruff, L. LL.: *Foundations of Biology.*

Open Court Publishing Co.: Brodetsky, S.: *First Course in Nomography*; Friess, Horace Leland: *Schleiermacher's Soliloquies*; Leathem, J. G.: *The Mathematical Theory of Limits*; Piaggio, H. T. H.: *Elementary Treatise on Differential Equations and their Application*; Silberstein, L.: *Projective Vector Algebra.*

Oxford University Press: de Beer, G. R.: *An Introduction to Experimental Embryology*; Dobson, G. M. B., Griffith, I. O. and Harrison, D. N.: *Photographic Photometry*; Goodrich, Edwin S.: *Living Organisms, an Account of their Origin and Evolution*; Heresy, George and Panetti, Fritz: *Manual of Radioactivity*; Hinshelwood, C. N.: *Kinetics of Chemical Change in Gaseous Systems*; Smith, G. Eliot: *The Evolution of Man*; Colwell, H. C.: *Introduction with Study of Roentgen Rays and Radium*; Cooper, Eugenia R. A.: *The Histology of the More Important Human Endocrine Organs at Various Ages*; Dakin, W. J.: *The Elements of General Zoology*; Dodds, E. C. and Dickens, F.: *The Chemical and Physiological Properties of the Internal Secretions.*

Presses Universitaires de France: *Problèmes Biologiques*, 4–6.

Princeton University Press: Conklin, E. G.: *A Synopsis of the General Morphology of Animals*; More, Louis T.: *The Dogma of Evolution*; Morgan, T. H.: *Evolution and Genetics.*

W. B. Saunders Co.: Arey, Leslie Brainerd: *Developmental Anatomy*; Castle, W. E. et al: *Our Present Knowledge of Heredity*; Cecil, R. L.: *A Text-book of Medicine by American authors*; Dorland, W. A. N.: *American illustrated Medical Dictionary*; Falk, I. S.: *Principles of Vital Statistics*; Friedenwalt, J.: *Diet in Health and Disease*; Herrick, C. J.: *Neurology*; Herrick, C. Judson: *An Introduction to Neurology*; Kolmer, John A.: *A Practical Text-book of Infection, Immunity and Biological Therapy*; Stollmann, Torald: *A Manual of Pharmacology*; Stengel, Alfred and Fox, Herbert: *Text of Pathology*; Stevens, A. A.: *The Practice of Medicine*; Stiles, Percy: *Human Physiology*; Todd, J. C.: *Clinical Diagnosis by Laboratory Methods*; Wells, H. Gideon: *Chemical Pathology.*

D. Van Nostrand Co.: Baker, A. L.: *Thick Lens Optics*; Brownell, B.: *The New Universe*; Cathcart, W. L. and Chaffee, J. I.: *The Elements of Graphic Statics*; Howe, Harrison E.: *Chemistry in the World's Work*; Lee, Willit, T.: *Stories in Stone*; Troland, L. T.: *The Mystery of Mind.*

Yale University Press: Lewis, Gilbert N.: *The Anatomy of Science*; Millikan, R. A.: *Evolution in Science and Religion.*

From the authors: *Bailey's Text-book of Histology,* revised by O. S. Strong and Adolph Elwyn; Conklin, Edwin G.: *A Synopsis of the General Morphology of Animals*; Curtis, Winterton C.: *Textbook of General Zoology* (2 copies); Harvey, E. Newton: *Laboratory Directions in General Physiology*; Herrick, C. Judson: *Brains of Rats and Men*; Johnson, Chas W.: *The Insect Fauna (Biological Survey of the Mt. Desert Region)*; Mathews, A. P.: *The Nature of Matter, Gravitation and Light*; McKeough, Rev. Michael J.: *The Meaning of the Rationes Seminales in St. Augustine*; Means, James H.: *Dyspnoea*; Patten, Bradley M.: *The Embryology of the Pig*; Pratt, H. S.: *A Laboratory Course in General Zoology*; Warbasse, James P.: *Surgical Treatment*; Wieman, H. L.: *General Zoology*; Woodruff, L. L.: *Foundations of Biology*; Workman, F. B. and Workman, W. H.: *Algerian Memories*; *The Call of the Snowy Hispar*; *Ice-bound Heights of the Mustagh*; *Illustrations of Ice Wilds of Eastern Karakoram*; *In the Ice World of Himálaya*; *Peaks and Glaciers of Nun Kun*; *Sketches Awheel in Fin de Siècle Iberia*; *Through Town and Jungle*; *Two Summers in the Ice-Wilds of Eastern Karahoram.*

An especially notable gift this year is that of the Library on Coelenterates of Professor Charles Wesley Hargitt, presented to the Library of the Marine Biological Laboratory by his son, Professor George T. Hargitt. The volumes and pamphlets number about 500 in all. They will not be catalogued and shelved as a special library, but each will be marked by a special bookplate, and in addition, the collection as a whole will be marked by a small brass tablet with an inscription showing that it is incorporated with the other books of the Library. This commemoration tablet, in size about 7″ x 9″, will be placed on the walls in the stack-room of the reading-room floor.

WOODS HOLE
LIBRARY

A special acknowledgment should be made of a gift of reprints, books and pamphlets sent by Dr. Ida H. Hyde who had already presented to this Library in 1917–18, the main collection of her reprints. The library wishes to acknowledge another gift from Mrs. Edward G. Gardiner, of reprints from Dr. Gardiner's library. Dr. Louis Murbach has presented a number of his books and pamphlets that will be of use in the Library here. And very especially, the Library makes acknowledgment of a gift of books and other interesting pamphlets which were sent to us by Dr. Elizabeth H. Dunn when she closed her shop in Woods Hole.

The sum granted by the General Education Board for back sets was $5000 more this year than in 1926, and the number of sets completed for the library was, therefore, correspondingly larger. 84 back sets were completed and 30 partially completed. The most interesting of these are:

Periodicals: *Jahrbücher für wissenschaftliche Botanik*; *Journal of the Franklin Institute*; *Philosophical Transactions of the Royal Society of London*; *Proceedings of the Zoölogical Society of London*; *Transactions of the Zoölogical Society of London*; *Zeitschrift für wissenschaftliche Zoologie*; *Zoologica.*

Serially issued monographs: *Bijdragen tot de Dierkunde K. zoologisch genootschap Natura Artis Magistra te Amsterdam*; Oppel, Albert: *Lehrbuch der vergleichenden Mikroskopischen Anatomie der Wirbeltiere*; Oppenheimer, C.: *Handbuch der Biochemie des Menschen und der Tiere*; Chun, Carl: *Valdivia Expedition.*

The completion of back sets of serials has not been confined to those purchased. We owe grateful acknowledgment for 7 sets completed by gift and 19 partially completed. The back sets of the Biological Bulletin have also been used to secure missing sets of serials; 19 having been completed by this method, and 21 partially filled in. In this connection, there should be mentioned also the use made of the duplicate material which was listed and arranged alphabetically in 1925–26. Several important gaps have been filled through exchange for these duplicates. It is greatly to be regretted that lack of time, both on our part, and the part of other libraries, hinders a freer advertisement of duplicate material.

The financial statement and the acquisitions of the year are simple to enumerate. The uses made of the Library during the year cannot so easily be appraised. This is especially true beginning with this year when new books and the serial publications were for the first time restricted in place of use to the Library itself. 1,846 loans were charged out, however, during the year. The out-of-town loans were greater than in any previous year, although but 11. Also more volumes were borrowed from other libraries, 37 in all, one more than in 1926 and five more than in 1925.

VI. THE REPORT OF THE DIRECTOR.

To the Trustees of the Marine Biological Laboratory.

Gentlemen: I beg to submit herewith a report of the fortieth session of the Marine Biological Laboratory for the year 1927.

1. *Attendance.*—In the Tabular View of Attendance on page 41 a departure has been made from the previous customs of listing the investigators under the three headings of Zoölogy, Physiology and Botany. This change has appeared advisable, first, because the broadening of the activities of the Laboratory in recent years has brought to it an increasing number of persons whose work does not, strictly speaking, fall into any of these categories, and, second, because a considerable number of investigators in filling out their registration blanks have indicated that in their own opinion their work belongs equally to Zoology and Physiology or to Botany and Physiology. For this reason investigators have been classified merely as "independent" or "under instruction." Following the custom inaugurated in 1926 a separate class has been provided for research assistants, whose number during the past few years has been rapidly increasing.

An examination of the figures for the attendance during the years 1923–7, inclusive, shows that the number of students in the courses has remained nearly stationary owing to the strict limitation of the sizes of our classes. The attendance of investigators, on the other hand, being subject to no such restriction has shown a remarkable growth. For 1927 the increase over the preceding record-breaking year of 1926 was approximately thirteen per cent. This increase is especially remarkable in view of the fact that for a considerable time during the previous summer every room in both the brick and the wooden buildings was occupied. The accommodation of over 40 additional investigators under these circumstances was made possible, first, by the conversion into laboratories of several rooms formerly used for other purposes, second, by an extensive sharing of the larger rooms by two or more workers, and, third, by a considerable lengthening of the season of greatest activity. To encourage the attendance of investigators during the less crowded parts of the summer, the Mess in 1927 was opened about two weeks earlier and closed about five days later than in previous years. This policy has proved to be so successful that it is planned to continue it.

A consideration of the unprecedented increase in the number of investigators during the past two years, which considerably exceeds that for the previous thirteen, raises the question of the maximum capacity of the present laboratory buildings. It may be said in this connection that for the months of July and August the limit has already been almost, though not quite, reached. A further sharing of rooms during this period will make possible the accommodation of a small additional number of investigators. However, chief reliance in the future must be placed upon a further extension of the working season. With the Laboratory at present occupied to its full capacity for only two months of the year it is evident that it is still far from having reached the condition of its greatest usefulness.

The possibilities for extending the season in both directions are strikingly shown by the following tabulation of the numbers of investigators and research assistants in attendance on selected days throughout the summer of 1927:

April 20	None
" 30	1
May 10	3
" 20	6
" 30	7
June 10	50
" 20	114
" 30	212

July 10	247
" 20	247
" 30	245
August 10	234
" 20	208
" 30	168
September 10	110
" 20	50
" 30	12
October 10	8
" 20	2
" 30	2
November 10	None

2. *The New Dormitory and Apartment House.*—In the report of the Directors for 1926 mention was made of the generous gift of $250,000 by the General Education Board for the purpose of erecting a Dormitory and an Apartment House. Both of these buildings were completed, except for certain minor details, early in June, 1927. The first family moved into the Apartment House on June 1 and the first persons into the Dormitory a few days later. From that time until early in September the buildings were occupied to almost their full capacity and were not entirely vacant until the first of November.

The new buildings fill admirably the long-felt need of the Laboratory for suitable accommodations for investigators with families. Together they provide six large apartments, fully equipped for housekeeping, each consisting of a living-room, two bed-rooms, screened porch, kitchenette, and bath; two smaller apartments of similar character but with only one bed-room and without a porch; nine suites of two rooms with bath; sixteen double rooms which can be combined in various ways into suites; nineteen other double rooms and eighteen single rooms. Each of the rooms which is not part of a suite containing a bath is provided with hot and cold running water. For the use of those persons who do not occupy the furnished apartments there is provided in each of the buildings a large and comfortable social room and in the basement facilities for laundry work and simple cooking. An especially valuable feature of the Apartment House is that it can be heated,

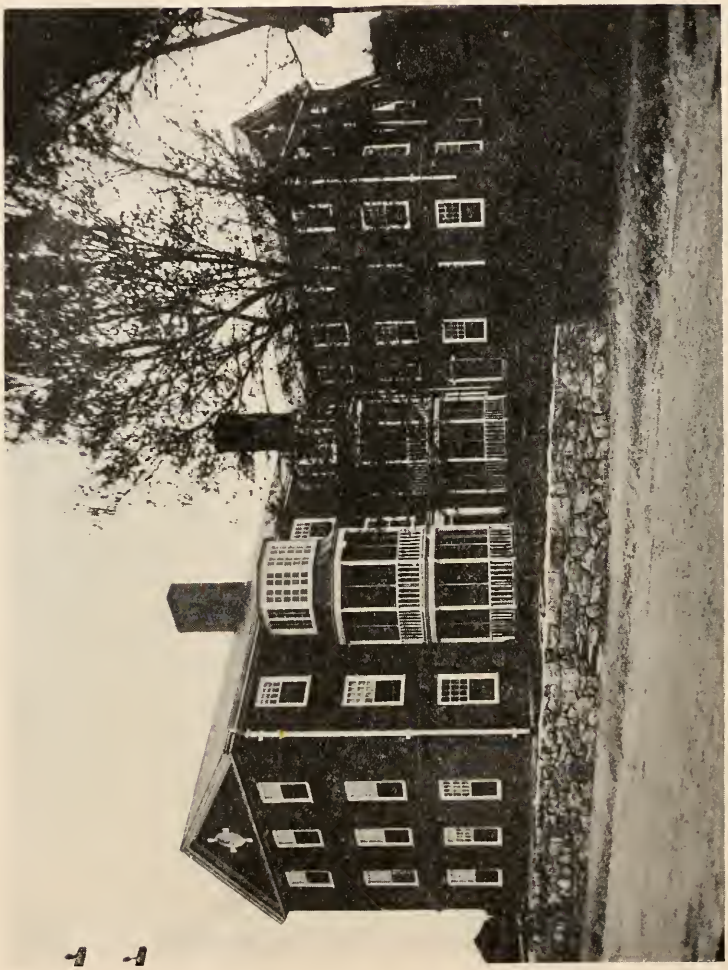

Fig. 1. The New Apartment House.

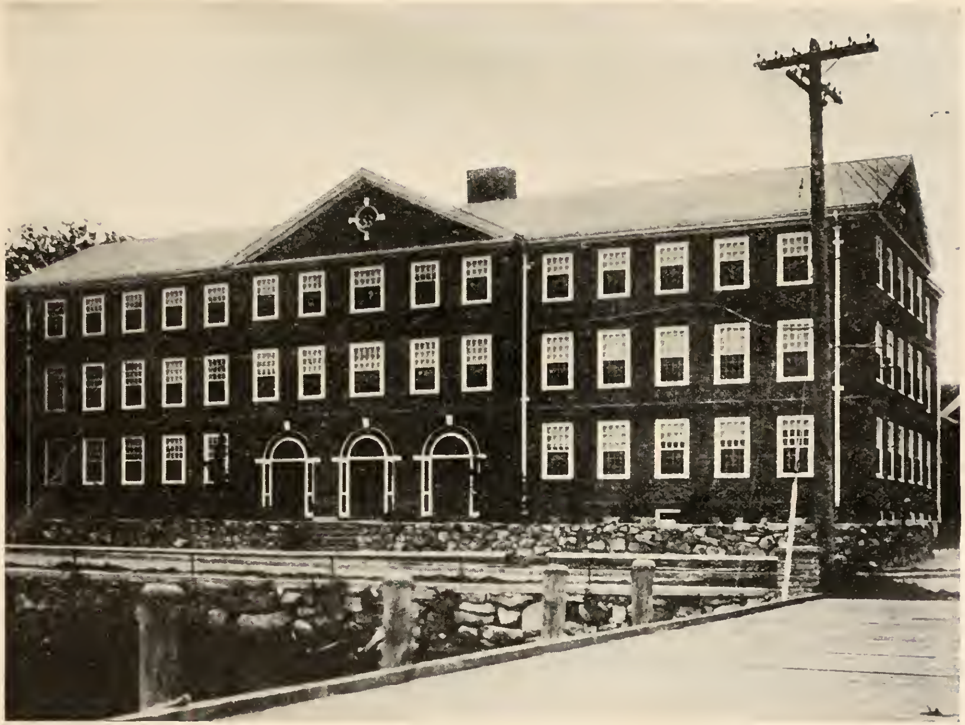

FIG. 2. The New Dormitory.

thus making it possible for the working season of the Laboratory to be extended into the colder months of the year.

According to the original plans, the Apartment House was to be used for investigators and the Dormitory for students, but the experience of the first year of operation has shown that the demand from the investigators alone will be more than sufficient to fill both buildings, and, on the whole, it seems fairer to give preference in the assignments to this class of workers, both because their stay at the Laboratory is longer and because they have greater difficulty on account of their families in finding suitable accommodations in the village. The usefulness of the new buildings to investigators with families is indicated by the fact that during the first year of their operation they accommodated a total of 38 children.

The following is a classification of the occupants of the two buildings for 1927:

	Dormitory	Apartment House	Total
Investigators	52	28	80
Members of familes of investigators	47	41	88
Students	7	–	7
Total	106	69	175

The cost of the buildings, in spite of various unforseen difficulties which arose during their construction has to the time of this report come well within the appropriation of $250,000, though a certain amount of interior painting still remains to be done. This very favorable financial showing would have been impossible, however, without the generous assistance of Dr. Frank R. Lillie in connection with the grading of the grounds and the planting of shrubbery which have added greatly to the appearance of the buildings. The following figures taken with slight simplification from the Auditors' Report for 1927 show the status of the building fund on December 31, 1927:

ANALYSIS OF COST OF DORMITORY BUILDING AND APARTMENT HOUSE

	Dormitory	Apartment House
General Contractor	$ 90,016.70	$ 72,086.88
Plumbing	16,451.00	13,170.00
Electrical Work	4,657.98	5,378.73
Heating	649.00	5,800.00
All other Building Expenses	14,806.75	13,185.18
Total Building Expenses	$126,581.43	$109,620.79
Furnishings	7,266.26	6,067.33
Total	$133,847.69	$115,688.12

Summary

Total New Dormitory	$126,581.43	
" Apartment House	109,620.79	$236,202.22
New Dormitory Equipment	7,266.26	
Apartment House Equipment	6,067.33	13,333.59
Total Cost to Dec. 31, 1927		$249,535.81
Accounted for as follows:		
Gifts of General Education Board	$250,000.00	
Less Cash on Hand	2,898.31	
	$247,101.69	
Gift of Dr. Frank R. Lillie for Grading, etc.	1,500.00	
Payments from Current Funds	251.57	
Unpaid Balance of Architects' Commission	682.55	$249,535.81

3. *Other Buildings.*—Among the additions to the permanent equipment of the Laboratory during 1927, one which would have received more notice than it has in a year not marked by such extensive building operations is the new carpenter shop and boat house, situated beside the Eel Pond near the Marine railway.

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WOODS HOLE
LIBRARY
MASS.

The erection of this building involved the previous preparation of the site by extensive filling with material from the excavations for the Dormitory and the construction of a substantial retaining wall. The building itself measures 66 by 44 feet and is two stories in height. The first story includes a carpenter shop 33 by 44 feet and a boat shop of the same size; the latter is on the side of the building adjacent to the Marine railway and is provided with doors of sufficient size to admit large boats. The second story consists of a single large room, used at present chiefly for storage purposes but suitable for meeting a variety of needs which may arise in the future. The cost of the building with the retaining wall was approximately $7,500.

While the new Dormitory and Apartment House were under construction the Dexter House, which for some years has accommodated a large number of our students and younger investigators was removed. Many of its former occupants were cared for during the past summer in the new buildings and an additional number in the Drew House, whose capacity has been considerably increased by a more economical use of space, and which is now used exclusively as a men's dormitory.

4. *The Report of the Treasurer* shows an increase in the total assets of the Laboratory from $2,281,219.79 in 1926 to $2,449,624.06 in 1927, the largest single item in this increase being accounted for by the completion during the past year of the new dormitory and apartment house buildings. The income for 1927 was $193,707.42 and that shown in the Auditors' Report for 1926 was $187,979.11—an apparent increase of approximately $6000. The actual increase, however, was in reality nearly $16,000, since the gift of $10,000 from the General Education Board in 1926 was listed under income and the corresponding gift of $15,000 in 1927 under current surplus. By the same system of bookkeeping as that adopted in 1927 the income for the previous year would be $177,979.11 instead of the figure mentioned above. The expenses for the two years were $180,182.80 in 1926 and $193,798.48 in 1927, giving apparent deficits of $2,203.69 and $91.06, respectively. These deficits are at present on paper only and are due to the depreciation charged against the plant which the Treasurer discusses in his report. It may be mentioned in

this connection that no charge for depreciation was made for 1927 in the case of the new Dormitory and Apartment House buildings, since some work still remained to be done upon them and the construction account had not therefore been closed into the general plant account. In future years, however, an additional annual depreciation charge of $5,000 on these buildings will, unless additional sources of income are found, still further increase the unfavorable balance of the past two years. In this connection it is well to remember that depreciation in the case of the Laboratory buildings occurs during twelve months of the year while most of the income derived from them is at present limited to three months. This is an additional reason for making every effort to increase the length of the active season of the Laboratory.

5. *The Report of the Librarian* shows a very substantial increase in our library facilities during the past year. The greater part of this increase has been made possible by a second installment of $15,000 from the gift of $50,000 appropriated by the General Education Board in 1925. This sum was used chiefly for the purchase of back sets of serials and the Librarian was fortunate in being able to fill some of the most serious of the existing gaps in these sets. Another noteworthy addition to the library during 1927 was the valuable collection on Coelenterates of the late Professor Charles W. Hargitt, generously presented to the Laboratory by his son, Professor George T. Hargitt. The total number of bound volumes on our shelves at present is over 22,000 and of reprints over 43,000. Of the former, approximately 4,000 and of the latter approximately 5,000 were acquired during the past year. The number of current serials regularly received by the Library is now nearly 800.

6. *The Loeb and Gardiner Memorial Tablets.*—On August 4 there was unveiled a tablet to Jacques Loeb, whose work, carried on at Woods Hole over a period of more than twenty years, has been one of the outstanding contributions to science of the Marine Biological Laboratory. Addresses were delivered in this occasion by Professor Frank R. Lillie, Doctor Simon Flexner and Professor Hardolph Wasteneys.

The tablet bears the following inscription:

JACQUES LOEB
1859 — 1924
BRAIN PHYSIOLOGY
TROPISMS, REGENERATION
ANTAGONISTIC SALT ACTION
ARTIFICIAL PARTHENOGENESIS
DURATION OF LIFE
COLLOIDAL BEHAVIOR

A similar tablet was unveiled on September 10 to Edward Gardiner Gardiner, one of the founders of the Laboratory and until his death one of its most loyal supporters. Addresses were delivered by Professor E. G. Conklin and Professor Frank R. Lillie.

The tablet is inscribed as follows:

EDWARD GARDINER GARDINER
ZOOLOGIST
INCORPORATOR OF THE MARINE BIOLOGICAL LABORATORY
AND MEMBER OF THE ORIGINAL BOARD OF TRUSTEES
FOR MANY YEARS CLERK OF THE CORPORATION AND
SECRETARY OF THE BOARD OF TRUSTEES
A MAN WHOSE FINE SENSE OF HONOR AND LOYALTY
SHONE IN HIS DEEDS
A SCIENTIST TO WHOM THE SUCCESS OF THE INSTITUTION
WAS THE FULFILLMENT OF HIS LIFE INTEREST
1854 — 1907

7. *Changes in Personnel.*—At the close of the courses in 1927 the resignation of Dr. Ivey F. Lewis, who for twenty years has been in charge of the Botany Course, was received and accepted with regret. The valuable services to the Laboratory of Dr. Lewis were recognized in the following resolution of the Board of Trustees:

Voted: That the Board of Trustees learns with regret of the resignation of Doctory Ivey F. Lewis from the headship of the course in Botany at the Marine Biological Laboratory, and expresses to Doctor Lewis its keen appreciation of his highly efficient and constructive conduct of the work during his long service of twenty years.

Dr. Lewis' successor is Dr. W. R. Taylor, Professor of Botany at the University of Pennsylvania, who both by his scientific attainments and by his long experience with the work of the course in excellently fitted for the position.

8. *The Board of Trustees.*—At the annual meeting of the Board of Trustees held August 9, 1927, Professor B. M. Duggar of the University of Wisconsin was elected to fill a vacancy in the Class of 1931 of the Board.

There are appended as parts of this report:

1. The Staff, 1927.
3. Investigators and Students, 1927.
3. A Tabular View of Attendance, 1923–1927.
4. Subscribing and Cooperating Institutions, 1927.
5. Evening Lectures, 1927.
6. Members of the Corporation, August, 1927.

1. THE STAFF, 1927.

Merkel H. Jacobs, *Director,* Professor of General Physiology University of Pennsylvania.

Associate Director: ———— ————.

I. INVESTIGATION.

Gary N. Calkins, Professor of Protozoölogy, Columbia University.

E. G. Conklin, Professor of Zoölogy, Princeton University.

Caswell Grave, Professor of Zoölogy, Washington University.

H. S. Jennings, Professor of Zoölogy, Johns Hopkins University.

Frank R. Lillie, Professor of Embryology, The University of Chicago.

C. E. McClung, Professor of Zoölogy, University of Pennsylvania.

S. O. Mast, Professor of Zoölogy, Johns Hopkins University.

T. H. Morgan, Professor of Experimental Zoölogy, Columbia University.

G. H. Parker, Professor of Zoölogy, Harvard University.

E. B. Wilson, Professor of Zoölogy, Columbia University.

Lorande L. Woodruff, Professor of Protozoölogy, Yale University.

II. INSTRUCTION.

J. A. Dawson, Instructor in Zoölogy, Harvard University.

Rudolf Bennitt, Instructor in Biology, Tufts College.

E. C. Cole, Assistant Professor of Zoölogy, Williams College.

T. H. Bissonnette, Professor of Biology, Trinity College.

Madeleine P. Grant, Assistant Professor of Zoölogy, Mount Holyoke College.

E. A. Martin, Assistant Professor of Zoölogy, College of the City of New York.

A. E. Severinghaus, Instructor in Zoölogy, Columbia University.

Donnell B. Young, Associate Professor of Biology, University of Arizona.

PROTOZOÖLOGY.

I. INVESTIGATION.

(*See Zoölogy.*)

II. INSTRUCTION.

Lorande Loss Woodruff, Professor of Protozoölogy, Yale University.

Gary N. Calkins, Professor of Protozoölogy, Columbia University. (Absent in 1927.)

Mary Stuart MacDougall, Professor of Zoölogy, Agnes Scott College.

W. B. Unger, Assistant Professor of Zoölogy, Dartmouth College.

Mary Stuart MacDougall, Professor of Zoölogy, Agnes Scott College.

EMBRYOLOGY.

I. INVESTIGATION.

(*See Zoölogy.*)

II. INSTRUCTION.

Hubert B. Goodrich, Professor of Biology, Wesleyan University.

Benjamin H. Grave, Professor of Biólogy, Wabash College.

Charles Packard, Associate in the Institute of Cancer Research, Columbia University.

Harold H. Plough, Professor of Biology, Amherst College.

Charles G. Rogers, Professor of Comparative Physiology, Oberlin College.

PHYSIOLOGY.

I. INVESTIGATION.

Harold C. Bradley, Professor of Physiological Chemistry, University of Wisconsin.

Walter E. Garrey, Professor of Physiology, Vanderbilt University Medical School.

Ralph S. Lillie, Professor of General Physiology, The University of Chicago.

Albert P. Mathews, Professor of Biochemistry, The University of Cincinnati.

II. INSTRUCTION.

Merkel H. Jacobs, Professor of General Physiology, University of Pennsylvania.

Wallace O. Fenn, Professor of Physiology, University of Rochester.

Leonor Michaelis, Professor in the University of Berlin and Resident Lecturer in the Johns Hopkins Medical School.

H. K. Hartline, Department of Physiology, Johns Hopkins University.

Charlotte Haywood, Department of Physiology, University of Pennsylvania.

BOTANY.

I. INVESTIGATION.

B. M. Duggar, Professor of Plant Physiology, Washington University.

C. E. Allen, Professor of Botany, University of Wisconsin.

S. C. Brooks, Department of Public Health, Washington, D. C.

Wm. J. Robbins, Department of Botany, University of Missouri.

J. R. Schramm, Editor-in-Chief; Biological Abstracts, University of Pennsylvania.

II. INSTRUCTION.

Ivey F. Lewis, Professor of Biology, University of Virginia.

William Randolph Taylor, Assistant Professor of Botany, University of Pennsylvania.

James P. Poole, Professor of Evolution, Dartmouth College.

LIBRARY.

Priscilla B. Montgomery, (Mrs. Thomas H. Montgomery, Jr.), Librarian.

Katherine Underwood, Assistant Librarian.

Deborah Lawrence, Secretary.

CHEMICAL SUPPLIES.

Oliver S. Strong, Associate Professor of Neurology, Columbia University, *Chemist.*

APPARATUS ROOM.

Samuel E. Pond, Assistant Professor of Physiology, Medical School, University of Pennsylvania, *Custodian of Apparatus.*

SUPPLY DEPARTMENT.

George M. Gray, Curator.
Assistant Curator: —— ———.
John J. Veeder, Captain.
E. M. Lewis, Engineer.
A. W. Leathers, Head of Shipping Department.
A. M. Hilton, Collector.
J. McInnis, Collector.

F. M. MacNaught, Business Manager.
Herbert A. Hilton, Superintendent of Buildings and Grounds.
Thomas Larkin, Superintendent of Mechanical Department.
Raymond E. Phipp, Mechanician.
William Hemenway, Carpenter.
Arnold H. Bisco, Storekeeper and Head Janitor.

2. INVESTIGATORS AND STUDENTS, 1927.

Independent Investigators.

Addison, W. H. F., Professor of Normal Histology and Embryology, University of Pennsylvania.
Allee, W. C., Associate Professor of Zoölogy, University of Chicago.
Allen, Ezra, Research Associate, Carnegie Institution of Washington.
Amberson, William R., Assistant Professor of Physiology, University of Pennsylvania.
Armstrong, Philip B., Instructor in Anatomy, Cornell University Medical College.
Austin, Mary L., Lecturer in Zoölogy, Barnard College.
Baitsell, George A., Associate Professor of Biology, Yale University.
Baker, Lillian E., Assistant in Department of Experimental Surgery, Rockefeller Institute for Medical Research.
Belling, John, Investigator, Carnegie Institution of Washington.
Bennitt, Rudolf, Instructor in Zoölogy, Tufts College.
Berrill, N. J., Assistant of Zoölogy Department, University College, London, England.
Bigelow, Robert P., Professor of Zoölogy and Parasitology, Massachusetts Institute of Technology.
Bissonnette, Thomas Hume, Professor of Biology, Trinity College.

Bowen, Robert H., Assistant Professor of Zoölogy, Columbia University.

Bradley, Harold C., Professor of Physiological Chemistry, University of Wisconsin.

Breitenbenbecher, Joseph K., Lecturer in Zoölogy, McGill University.

Bridges, Calvin B., Research Assistant, Carnegie Institution of Washington.

Bronfenbrenner, Jacques J., Associate Member, Rockefeller Institute.

Bronk, Detlev W., Assistant Professor of Physiology and Biophysics, Swarthmore College.

Brooks, Matilda Moldenhauer, Associate Biologist, Hygienic Laboratory, Washington.

Brooks, Sumner C., Professor of Physiology and Biochemistry, Rutgers University.

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Protozoölogy.

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3. TABULAR VIEW OF ATTENDANCE.

	1923	1924	1925	1926	1927
Investigators—Total	176	194	207	252	294
Independent	126	124	135	156	209
Under Instruction	50	70	72	84	57
Research Assistants				12	28
Students—Total	146	134	132	141	141
Zoölogy	59	50	54	56	57
Protozoölogy	16	17	17	19	17
Embryology	31	29	29	28	32
Physiology	22	18	19	18	19
Botany	18	20	13	20	16
Total Attendance	322	328	339	393	435
Less persons registered as both Students and Investigators				8	1
				385	434
Institutions Represented—Total	107	110	112	119	111
By investigators	62	69	74	84	89
By students	73	68	65	60	63
Schools and Academies Represented					
By investigators	–	–	1	–	1
By students	4	–	4	4	4
Foreign Institutions Represented					
By investigators	–	–	–	17	15
By students	–	–	–	3	8

4. SUBSCRIBING AND COOPERATING INSTITUTIONS, 1927.

Amherst College
Antioch College
Barnard College
Bowdoin College
Brown University
Bryn Mawr College
Butler College
C. R. B. Educational Foundation
Carnegie Institution, Cold Spring Harbor
Carnegie Institution of Washington
Columbia University
Commonwealth Fund
Connecticut College
Cornell University
Cornell University Medical College
Dalhousie University
Dartmouth College
De Pauw University
Duke University
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5. EVENING LECTURES, 1927.

Tuesday, June 28,
Professor S. O. Mast.........."Structure, Locomotion and Stimulation in Ameba Proteus."

Friday, July 1,
Professor L. V. Heilbrunn...."The Colloid Chemistry of Protoplasm."

Tuesday, July 5,
Professor Alexander Forbes..."Implications of the All-or-none Principle in the Physiology of the Central Nervous System."

Friday, July 8,
Professor B. M. Duggar......."The Physiological Behavior of Certain Virus Agencies."

Tuesday, July 12,
Professor E. G. Conklin......"Localization Phenomena in Embryology."

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Friday, July 15,
Dr. E. M. Landis ...."The Permeability of the Capillary Wall."

Tuesday, July 19,
Dr. Jacques Bronfenbrenner.."The Nature of the Bacteriophage Phenomenon."

Friday, July 22,
Professor Frank R. Lillie....."The Gene and the Ontogenetic Process."

Tuesday, July 26,
Professor R. M. Yerkes........"The Psycho-biology of the Gorilla."

Friday, July 29,
Dr. Henry B. Bigelow........."Oceanographic Problems and Opportunities."

Tuesday, August 2,
Professor J. C. Drummond....."The Chemistry of Vitamines."

Wednesday, August 3, (Special Lecture)
Professor H. D. Fish.........."Life along the Trails and Streams of British Guiana."

Friday, August 5,
Dr. William Mansfield Clark."A Restricted but New Approach to Oxidation-reduction in the Living Cell."

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Zoölogical Laboratory, University of Pennsylvania.

Introduction.

The contractile vacuolar apparatus of Ciliata seems to be more highly specialized than that of other Protozoa. As to the form of the vacuole itself, we may recognize for convenience two general types among the Ciliata: (*a*) Vesicle-fed vacuoles, in which the contracting vacuole is surrounded by a series of small vacuoles (called vesicles in this paper) which seem to coalesce and form a new contracting vacuole after systole, new smaller vesicles appearing around the contracting vacuole after systole, and growing larger during diastole as described by Taylor ('23) in *Euplotes;* (*b*) Canal-fed vacuoles, in which the vacuole is fed by canals, which vary in number from one in *Spirostomum* and *Stentor* (Roux, '01) to thirty in *Ophryoglena* (Bütschli, '87–'89). A condition which seems to be intermediate has been described by Schewiakoff ('89) for *Prorodon teres.* Here the vacuole is fed by four radial rows of vesicles which decrease in size distally from the contractile vacuole.

The contractile vacuole usually communicates with the exterior by a tubule through a pore. This excretory tubule leading from the vacuole to the pore may be very short as in *Nassula* and *Frontonia* relatively long as in *Urocentrum,* or very long as in *Lembadion* (Schewiakoff, '89) where the vacuole is located near the central part of the body and the excretory tube leads to the pore which is about one quarter of the body length posterior to the vacuole.

The purpose of this paper is to describe the contractile vacuolar apparatus of *Paramecium trichium* which has been described by Stokes ('88) as consisting of two vacuoles "close together, contracting quickly, the one beginning to again form almost before the completion of the other's systole" and by Wenrich ('26) who was unable to reach a satisfactory conclusion as to "whether the two main vacuoles empty alternately into the same outlet tube or whether there is an auriculo-ventricular relationship between them."

Without the use of methods introduced by Bresslau ('21) as modified in this paper, the structures to be described could not have been studied. I am also particularly indebted to Doctor D. H. Wenrich who first pointed out the need for further study of the contractile vacuoles of *Paramecium trichium.*

Materials and Methods.

The *Paramecium trichium* used in this series of observations were obtained from two sources in the vicinity of Swarthmore, Pa.: from the east branch of Crum Lynne Creek where they were found among the jelly of toads eggs and from the west branch of Stony Creek, the source of Wenrich's ('26) Swarthmore race. The material was first studied in mixed cultures but later pure lines were established. For purposes of comparison, *Colpidium colpoda* from the same sources was studied.

Observations on the contractile vacuolar apparatus of *Paramecium trichium* were first made on material prepared according to the methods described by Bresslau ('21) and later those described by Coles ('27). These methods consist essentially of drying the protozoa in concentrated solutions of stains, which are relatively low in toxicity. The dye is precipitated in and on surface structures such as the depressions from which cilia arise, the cilia themselves, the mouth and the cytopharynx. The routine used is essentially as follows: A small drop of concentrated culture of the protozoa to be examined is placed upon a perfectly clean glass slip and mixed with a similarly sized drop of the stain. The two are then mixed and spread evenly over the slip with a needle or a glass spreader. The slides are then allowed to dry in the air and are examined under oil or mounted in damar.

Bresslau ('21) has used the following staining solutions: (*a*) Three parts saturated aqueous solution China Blue to one part saturated aqueous solution Cyanosin (a mixture previously used in bacteriological technique) and (*b*) one cc. 10 per cent. aqueous solution Opal Blue to 4–6 drops 6.5 per cent. aqueous solution Phloxinrhodamin.

Coles ('27) has used numerous stains for Protozoa in the same manner, obtaining his best results with a saturated aqueous solution Nigrosin.

The stains recommended by Bresslau are very toxic to Paramecium trichium which are killed quickly and usually burst open before they become dry. However, with solutions of (*a*) 10 per cent. China Blue (Coleman Bell), (*b*) 10 per cent. Nigrosin (Coleman Bell or Grübler), (*c*) Mixture of equal parts of above, and (*d*) 10 per cent. Opal Blue (Coleman Bell), beautiful preparations have been made. The above methods have been placed in order of their apparent usefulness for the structures studied in *Paramecium trichium.*

Observations made on such dried preparations have been verified on material fixed with Bouin's or Schaudinn's and stained with hæmalum and on living animals in hanging drops of the culture medium or of the culture medium mixed with China Blue, Opal Blue or Nigrosin. *Paramecium trichium* will survive over two hours in such hanging drops containing stains and remain apparently typical during that time.

Observations.

There are two contractile vacuoles in *Paramecium trichium,* one located in the anterior and one in the posterior part of the animal (Wenrich, '26). When dried in the staining solutions described above the coloring matter collects in the contractile vacuolar apparatus sometimes penetrating into the vacuole itself. The contractile vacuole is seen to be connected with the exterior by a long convoluted tubule (Figs. 2, 3, 4 and 9) which terminates in a small pore (Figs. 1, 2 and 4) located between the longitudinal rows of cilia on the dorsal surface of the body opposite the mouth. The pore of the anterior vacuole and that of the posterior open to the exterior between the same or adjacent rows of longitudinal rows of cilia (Figs. 8, 15 and 14). The vacuoles themselves do not seem to be fixed in position in living animals but move about more or less in the endoplasm; this apparently is made possible by the presence of the convoluted tubule. The inner end of the tubule terminates in a cup-like valve (Figs. 7 and 9) with which the vacuole is in contact when undergoing systole. This cup-like valve has been observed both in prepared slides and in living animals; while it seems always to be in contact with the vacuole it may be exterior or interior, anterior or posterior in relation to the vacuole. In fact the whole structure seems to be continually in active movement. The proximal portion of the tubule shows the effect of increased pressure at the beginning of systole, tending to straighten out as does a coiled hose when water is first turned into it under pressure.

In living animals each of the contractile vacuoles appears to be made up of two vacuoles which contract alternately. However, if they be carefully compared with a typical vesicle-fed vacuole such as that of *Colpidium colpoda,* it is found that the two are fundamentally alike. Observations made with a stop-watch show that the time relations of various parts of the cycle of systole and diastole in the two species are quite different. The table gives a set of typical observations on *Paramecium trichium* and *Colpidium colpoda.* These observations were made upon a single individual of each species on the same slide. For the interval between contractions seven groups of three contractions each were timed in *Colpidium colpoda;* ten groups of three contractions each in *Paramecium trichium* (anterior vacuole). A number of separate observations of the time from beginning to end of systole were made on the same individuals.

TABLE I.

TIME RELATIONS IN SECONDS OF CONTRACTILE VACUOLES OF *Paramecium trichium* AND *Colpidium colpoda* AT 25° C.

	Time between Completion of Two Systoles.	Time for Systole.
Paramecium trichium	3.1	2.0
Colpidium colpoda	7.4	0.4

In *Colpidium colpoda* the vacuole empties rapidly (about 0.4 seconds); the small feeding vesicles gradually enlarge, coalesce into one vacuole which finally reaches its maximum size about seven seconds after the last contraction, in the meantime there have appeared more tiny feeding vesicles around its periphery. The vacuole then contracts rapidly and the cycle is repeated.

In *Paramecium trichium* the vacuole (Fig. 12*a*) empties slowly (about 2 seconds). As it contracts the feeding vesicles grow (Fig. 12*b*) and by the time the vacuole has half completed systole (Fig. 12*c*) the feeding vacuoles coalesce with an apparent passing of membranes over their surface marking their fusion [called by Taylor ('23) "vestiges of the adjacent walls" in *Euplotes*]. When systole is completed (Fig. 12*d*) the new contractile vacuole has reached its maximum size; as it comes in contact with the cup-like valve a membrane (as described by Wenrich, '26) appears to pass over its surface, and it begins to contract (Fig. 12*e*).

The two processes are fundamentally alike; in *Paramecium trichium* the systole of the old contractile vacuole and the diastole of the new occur almost simultaneously and are of approximately the same duration while in *Colpidium colpoda* they occur consecutively with systole very brief and diastole protracted.

SUMMARY.

1. There is an anterior and a posterior contractile vacuolar apparatus in *Paramecium trichium*.

2. Each is permanent and consists of feeding vesicles, contractile vacuole, excretory tube and pore.

3. The contractile vacuoles of *Paramecium trichium* are vesicle-fed, differing here from those of other well known species in the same genus which are canal-fed.

4. Diastole of the new vacuole is practically complete before the prolonged systole of the old is over. This gives the appearance of two vacuoles contracting alternately.

5. The excretory tube is long and convoluted with its flattened cup-like end in contact with the contractile vacuole.

6. The excretory tube opens as an excretory pore on the surface of the body opposite the mouth.

7. The pore of the anterior and that of the posterior apparatus are located between the same or adjacent longitudinal rows of cilia.

8. The long duration of systole may be correlated with the presence of the convoluted excretory tube.

9. The excretory tube and pore were first demonstrated by the use of Bresslau's relief staining method.

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Plate I.

Explanation of Figures.

Figs. 1–11. Contractile vacuolar apparatus of *Paramecium trichium*, dried in 10 per cent. China Blue unless otherwise stated × 1000.

1. Posterior apparatus of animal photographed for Fig. 13.

2. Anterior apparatus of animal photographed for Fig. 14.

3. Posterior apparatus.

4. Posterior apparatus.

5. Anterior apparatus.

6. Posterior apparatus of same animal as Fig. 5.

7. Posterior apparatus of animal photographed for Fig. 16.

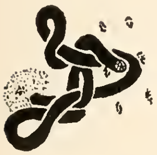
8. Entire animal showing cytopharynx, anterior and posterior vacuolar apparatus. Same as photograph Fig. 15.

9. Anterior apparatus.

10. Anterior apparatus (10 per cent. nigrosin).

11. Two tubules and pores from anterior end of same animal.

Fig. 12. Diagrams of various stages of contracting vacuole. *a.* Beginning of systole. *b.* Systole half-completed, feeding vesicles grow larger. *c.* Fusion of feeding vesicles. *d.* Systole complete. Cup-like inner end of tubule. *e.* Fusion of new contractile vacuole with cup-like inner end of tubule preparatory to systole.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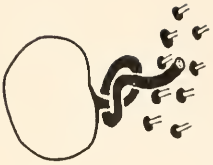
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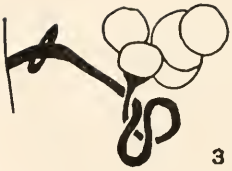
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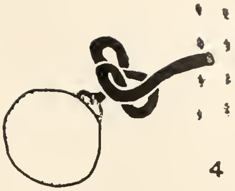
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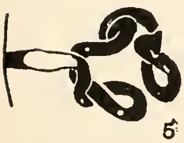
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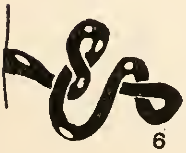
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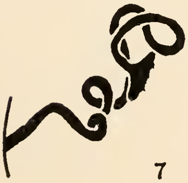
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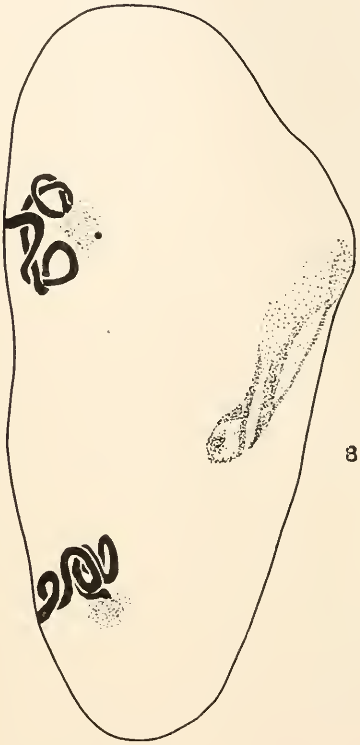
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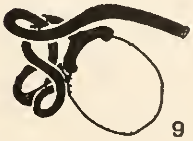
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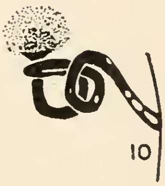
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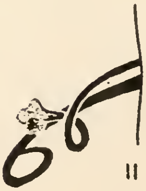
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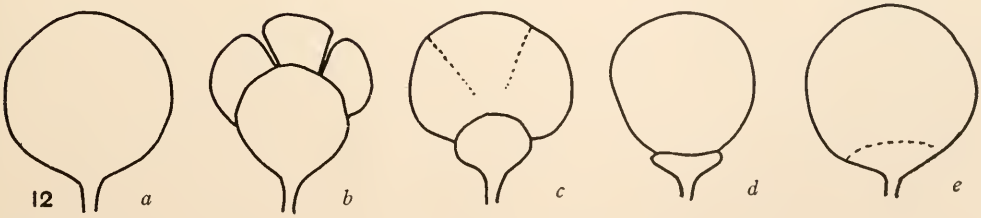
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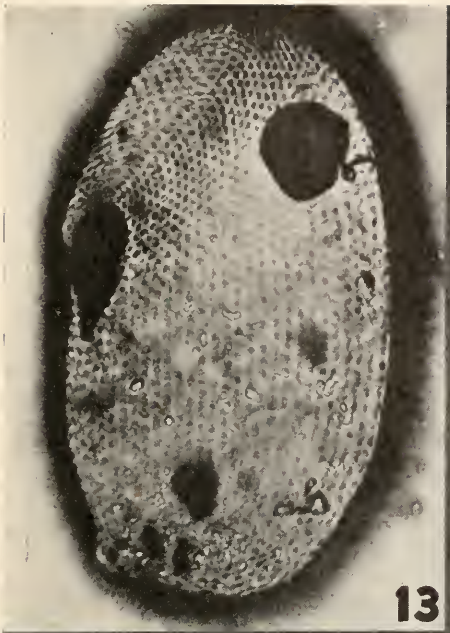
ROBERT L. KING.

Plate II.

Photographs × 750.

PLATE II.



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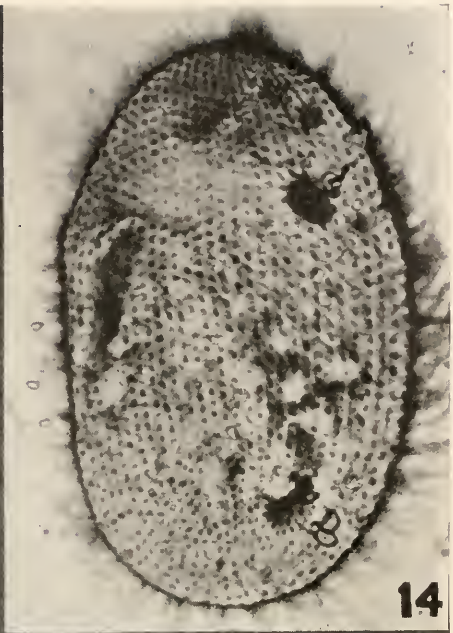

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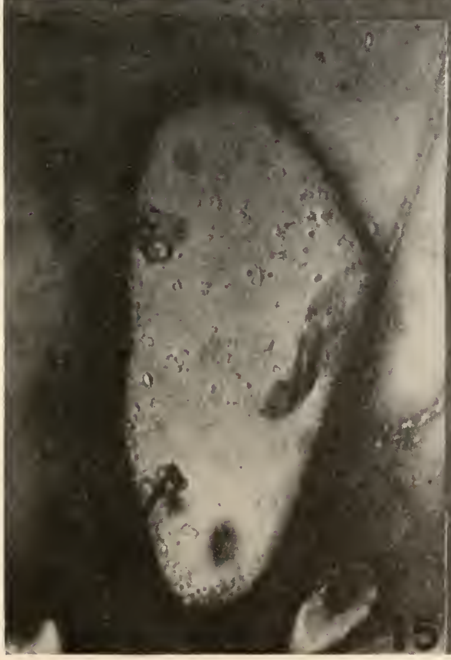

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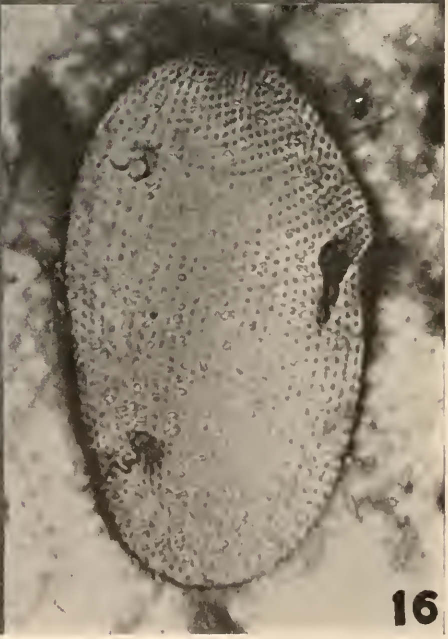

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ROBERT L. KING.

OBSERVATIONS OF THE FEEDING MECHANISM OF A CTENOPHORE, *MNEMIOPSIS LEIDYI*.[1]

ROLLAND J. MAIN.

Observations of the feeding habits of ctenophores are scattered through the literature dealing with these organisms (Bigelow, '15; Lebour, '22–'23; Mayer, '12; Nelson, '25), but as yet no detailed study of the feeding mechanism has appeared.

The ciliation of a hydromedusa has been studied (J. F. Gemmill, '19), but this compares in no way with the complex food catching apparatus of a ctenophore such as *Mnemiopsis leidyi.* The morphological work done upon this ctenophore is incomplete, for neither Agassiz (1849), Fewkes (1881), nor Mayer ('12), mention the presence of its remarkable mechanism for the capture of food.

Mnemiopsis leidyi through its habit of devouring the free-swimming larvæ of the oyster and of other molluscs becomes of such economic importance that it is of interest to determine by what means these organisms are captured and carried into the stomodæum, and how the undigested residues are discharged.[2]

MATERIALS AND METHODS.

The specimens of *Mnemiopsis leidyi* were obtained in the northern half of Barnegat Bay, N. J., a shallow estuary, in water of a specific gravity approximating 1.010, with temperatures close to 20° C., during August and the first part of September, 1926. The animals were caught in a net, placed in jars without injury and within ten minutes after capture they were being examined under the binocular.

Living plankton was used to determine the feeding mechanism, and it is felt that to this the success of the experiment is primarily due, since it is doubtful for reasons given below whether any other material could have been successfully used. The plankton was secured by pouring sea water through a 200 mesh plankton net, and concentrating the organisms in a small amount of water. A *Mnemiopsis* was placed in a watch crystal under the binocular, a little of the plankton culture was added, and the reactions of the ctenophore noted.

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[1] From the Zoölogical Laboratory of Rutgers University, Publication No. 11, New Jersey Oyster Investigation Laboratory.

[2] The writer is indebted to Dr. Thurlow C. Nelson of Rutgers University for aid and advice during this investigation and for reading the manuscript.

STRUCTURE AND OPERATION OF THE FOOD CATCHING MECHANISM.

To understand the mechanism of the food catching apparatus, it is first necessary to have a clear idea of the gross anatomy of *Mnemiopsis*, Fig. 1. Although considerable work has been done on the morphology of the animal, all the writers have apparently disregarded the presence of a definite ridge, an extension of the

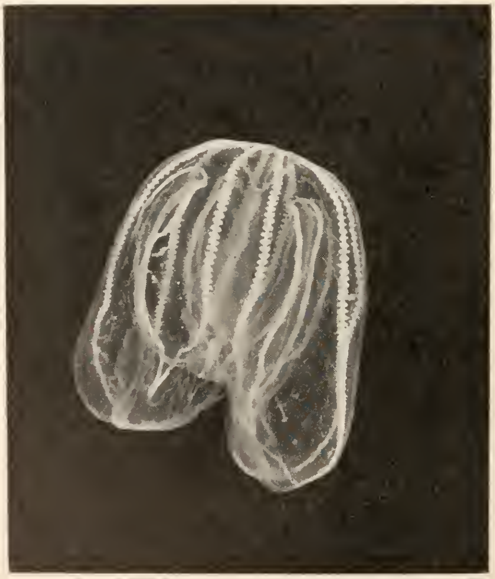

FIG. 1. Adult *Mnemiopsis leidyi* from Barnegat Bay. Photographed immediately after fixation in 10 per cent. hydrochloric acid. The oral lobes have contracted to approximately 2/3 the length characteristic of the living animal. Photographed by T. C. Nelson.

lips of the mouth, which the writer has named the "labial ridge." [3]

There are four furrows formed by the juncture of the oral lobes with the body. In each furrow along the inner side of the labial ridge is a line of tentacles. Through the base of this labial ridge runs a branch of the paragastric canal, which finally unites with the auricular canal. On the opposite side of this ridge is the ciliated channel for conveying food to the mouth, Figs. 2 and 3.

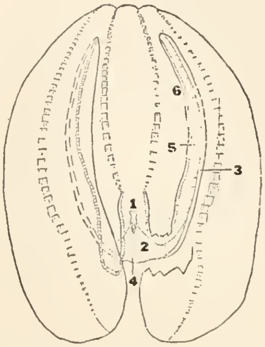

FIG. 2. *Adult Mnemiopsis leidyi.* Part of the right lobe and the tip of the right auricle have been omitted. It is difficult to represent the turning of the labial ridge. The lips are in the plane of the paper. As the lip becomes the labial ridge, it turns so that it lies in a plane at right angles to the paper. 1. The tentacular bulb. 2. The tentacular ridge, with tentacles. 3. The labial ridge, along which runs the tentacular ridge. 4. Lip. 5. Auricular groove. 6. Cilia of auricle.

To this channel, or trough, will be applied the term "labial trough." It is formed by the labial ridge on one side, and the oral lobe on the other. It runs along the ridge to the point where the ridge becomes the lip, and here the trough runs directly into the corner of the mouth, Fig. 4. The labial ridge is separated from the cilia of the auricles by the auricular groove in which the cilia of the auricle beat, and at the bottom of which lie the tentacles stretched out in the current.

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[3] The writer calls attention to some apparent discrepancies in earlier work on *Mnemiopsis leidyi.* Fewkes pictures an adult of this species which differs widely from the type obtained from Barnegat Bay. The latter, save for the contraction of the oral lobes, is well illustrated in Figure I. Fewkes' figure shows the surface of the animal covered with discoidal warts which are claimed by Mayer to be present in *M. mccradyi* and in *M. gardeni* but absent in *M. leidyi.* Fewkes' figure differs also in the shape of the body.

Near the mouth the line of tentacles curves away from the labial ridge up to the tentacular bulb. The tentacles are placed irregularly along this line, usually in groups, some animals having many more tentacles than others. This may be due to the fact that they have been broken off in securing food, for often food may be seen entering the stomodæum with portions of tentacles attached.

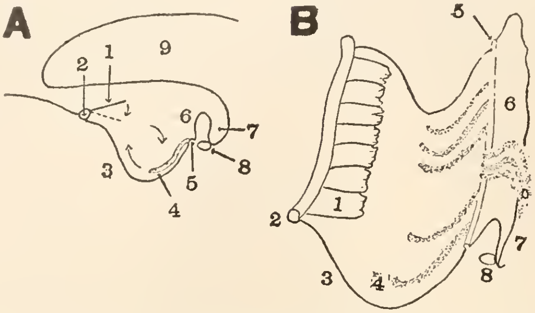

Fig. 3. *A*. Cross section of auricular groove. The cilia of the auricle (1) beat up and down as indicated by the arrow and dotted line. The other two arrows show the direction of the current produced by the cilia. 1. Cilia of auricle. 2. Auricular canal. 3. Auricular groove. 4. Tentacle. 5. Tentacular ridge. 6. Labial ridge. 7. Labial trough. 8. Branch of paragastric canal. 9. Oral lobe. *B*. View of auricular groove from above. The oral lobe has been laid back. Parts correspond to Fig. 3*A*. Three tentacles are here shown putting food in the labial trough, where it will be drawn off and conveyed to the mouth.

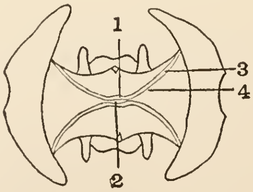

Fig. 4. Oral view of adult *Mnemiopsis leidyi*. This shows how the lips are continued into the labial ridge and how the trough runs into the mouth. 1. Lip. 2. Mouth. 3. Labial ridge. 4. Labial trough.

When a particle of food is caught in the current produced by the cilia in the grooves it is whirled about until it finally touches a tentacle. This entangles it, often with the aid of several other tentacles. These tentacles then contract, and apparently are drawn over the labial ridge into the labial trough, presumably by cilia, Fig. 3*B*. Here they stretch out in the direction of the mouth, the food is drawn off, and passes down toward the mouth. The tentacles then relax, and resume their normal position. Often several pieces of food are beaten about for some time in the groove. Dirt in the groove is gradually entangled in mucus into a long thread which slowly passes out at the aboral end of the groove. If much dirt be present, the whole animal pulsates, contracting the groove and forcing out all material present. The tentacles were never seen placing any foreign material into the labial trough, unless a little happened to be caught up with the food. Possibly it is for these reasons that *Mnemiopsis leidyi* is not found in muddy waters, since it will not seize food if much dirt be present. Carmine introduced directly into the labial trough is drawn along but for a short distance, and then is passed out over the labial ridge. For this reason the use of the natural plankton food organisms in studying the mechanism is imperative.

It is here that we must search for the explanation of why *Mnemiopsis leidyi* lives so largely upon bivalve larvæ, in spite of the great preponderance of other plankton in the water (Nelson, '25). The writer has observed that often the ctenophore is unable to hold an active copepod. Possibly the stronger swimmers are able to escape the ciliary currents, whereas the young oyster shuts its shell on contact and is therefore an easy prey. Polychæte larvæ were found in *Mnemiopsis* at this time, although never more than one or two per animal. This is contrary to Nelson's ('25) belief that it would be almost impossible for this ctenophore to ingest such a prey.

Food captured by the tentacles about the mouth was passed down directly over the lips into the mouth, often aided by a contraction of the lips, bringing them near the tentacular bulb. After the food has passed into the stomodæum, it usually proceeds slowly to the center, between the two paragastric canals, close to the convoluted tubules which probably secrete the digestive juices. It may, however, lodge below this point, Fig. 5. Sometimes it is caught in the swifter current at the very edge of the stomodæum, and is whirled up to the beating cilia at the aboral end. Here it is usually turned back, for these cilia seem to act partly as filters. At times, however, a particle may be squeezed through and enter the funnel to pass around in the food canals.

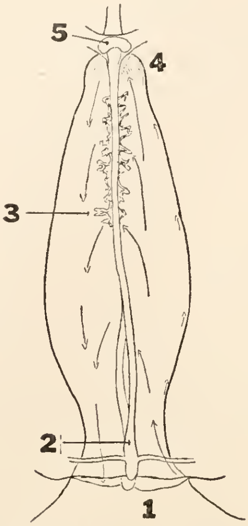

FIG. 5. The stomodæum of *Mnemiopsis leidyi.* In order to avoid confusion, the paths taken by ingested food are shown on the right side only. The larger arrows are the more usual paths. The smaller arrows on the extreme right denote a swifter current, in which the food sometimes travels. On the left half only, are shown the paths taken by the excreted materials. 1. Mouth. 2. Paragastric canal. 3. Digestive glands? 4. Cilia. 5. Funnel.

The undigested material in the stomodæum is passed down as indicated, and ejected through the mouth. These paths are not definite, for incoming food will pass a certain spot, and immediately afterwards outgoing wastes will cross the same spot going in the opposite direction. Those particles which have passed through into the food canals may reënter the stomodæum and pass out through the mouth, or they may follow the usual procedure for material in the canals, and be voided at the anus.

Just before defecation occurs, particles may be seen gathering about in the funnel and in the axial funnel canal. Then one of the branches of this canal elongates above the surface and the particles are forced out through the pore. The current in all the food canals now seems to be in the direction of the funnel. By this time the cilia of the aboral end of the stomodæum have ceased beating, and the whole upper end of the stomodæum presents a contracted appearance, Fig. 6. After the particles of waste have all passed out the cilia begin beating again, and the branch of the funnel canal slowly retracts. Although several successive defecations of specimens have been observed, only one branch was used, and in no specimen was the use of both branches observed.

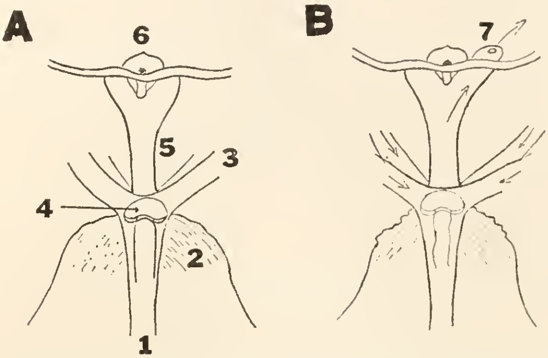

Fig. 6. Aboral portion of stomodæum, and axial funnel canal of *Mnemiopsis leidyi*. *A*. Before defecation. 1. Paragastric canal. 2. Cilia. 3. Food canals. 4. Funnel. 5. Axial funnel canal. 6. Sense organ. 7. Excretory pore. *B*. During defecation, arrows showing direction of waste. Note shrunken appearance of stomodæum.

The Early Development of the Food Catching Mechanism.

Since the complex food catching apparatus is present only in the adult *Mnemiopsis,* the question of its ontogeny naturally arises. The young were plentiful at the time of this study, and various stages were examined.

The smallest specimens obtained were in the Cydippidæ-stage, approximately 2 mm. high and 2 mm. broad, Fig. 7. These possess two long branching tentacles with no trace of the tentacular ridge, labial ridge, or labial trough. They feed by capturing the food with the tentacles, retracting them, and drawing them down over the lip and into the stomodæum, where the food is drawn off. Another contraction, and the tentacles emerge, to again float up above the animal.

The next step in development was found in a 6 mm. specimen, Fig. 8. This stage has still the two compound tentacles.

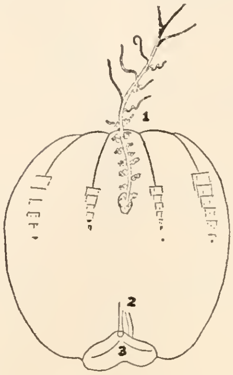

FIG. 7. Young *Mnemiopsis leidyi*, 2 mm. high. 1. Branching tentacle, partially contracted. 2. Paragastric canals, only unbranched terminations shown. 3. Mouth.

The 8 mm. specimens are much further advanced, Fig. 9. The auricles are now forming, and the tentacular ridge has appeared as a slight fold or line as shown, but it is not connected to the tentacular bulb, and possesses no tentacles. It was observed that tentacles never appeared along the tentacular ridge until it had joined the tentacular bulb.

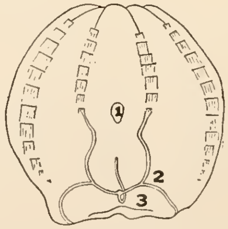

FIG. 8. Young *Mnemiopsis leidyi*, 6 mm. high. 1. Tentacular bulb. (Tentacle omitted, being same as in Fig. 7.) 2. Juncture of paragastric and auricular canals. 3. Mouth.

It is now easy to see how the adult structures are completed. As the junction of the paragastric and auricular canals moves upward forming the auricular groove, the tentacular ridge and labial ridge grow with it. The large branched tentacle disappears, and small tentacles appear along the tentacular ridge.

This remarkable food catching apparatus of *Mnemiopsis,* in which the conveying system seems to foreshadow that of the bivalves, is certainly a great advance over that of the Scyphozoa.

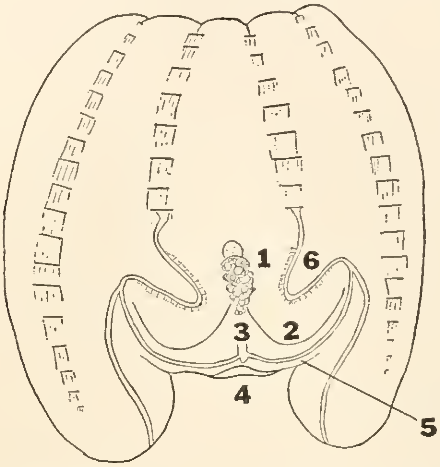

Fig. 9. Young *Mnemiopsis leidyi,* 8 mm. high. 1. Branched tentacle entirely retracted, but same as in Fig. 7. 2. Tentacular ridge. 3. Paragastric canal, termination shown with branches. 4. Mouth. 5. Beginning of labial ridge. 6. Developing auricles.

Of its efficiency there can be no doubt, for compare Bigelow's ('15) statement that the plankton was greatly diminished in a swarm of ctenophores. Nelson ('25) also brings forth evidence of a correlation between the abundance of *Mnemiopsis leidyi* and the intensity of shipworm infestation and oyster sets. Moreover, the fact that the ctenophores are usually found in such vast and dense swarms, argues well for their ability to obtain food. Possibly it is due to this efficient apparatus that we find in many species of ctenophores the small compact bodies and absence of long trailing tentacles.

Summary.

The mode of feeding was studied in young tentacled forms and in the adult *Mnemiopsis leidyi.* The young capture food with their branched tentacles, and deposit it in the mouth. The adults entangle the food with the small tentacles along the tentacular ridge, and deposit it in the labial trough, whence it is carried to the mouth.

Food enters the stomodæum and after digestion is cast out of the mouth, or it may enter the food canals and pass out of the anus.

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THE INFLUENCE OF OXYGEN TENSION UPON THE RESPIRATION OF UNICELLULAR ORGANISMS.

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Our knowledge of the influence of oxygen tension upon the oxygen consumption of unicellular organisms is quite incomplete. The literature contains many studies of the influence of oxygen tension changes upon growth and activity of such forms, but relatively few direct measurements of oxygen consumption have been made. In some studies in which the consumption has been measured the problem has been complicated by changes in the number of respiring cells during the course of the experiment. This would appear to be true of such observations as those of Stephenson and Whetham (1924) who have found that the oxygen intake of *B. coli* is much greater in pure oxygen than in air, and of Novy and Soule (1925) who report that the tubercle bacillus grows best in an atmosphere containing 40–50 per cent. oxygen, the growth and the oxygen consumption falling off progressively above and below this value. It is not possible to infer that a change in division rate indicates a change in the oxygen intake of the individual bacterium. The influence of the oxygen tension may be more indirect, possibly through the formation of such growth-promoting substances as Burrows (1924) has described, whose production is increased by an increased oxygen supply.

In other studies of bacterial respiration in which there has probably been no significant change in the number of respiring cells, Pütter (1924) and E. N. Harvey (1926) have secured evidence that the respiratory rate is not influenced by changes in the oxygen tension. In unicellular animal organisms the weight of the somewhat meagre evidence so far secured indicates that oxygen consumption is independent of oxygen tension over a wide range. Lund (1918) found this to be true for *Paramecium*. Henze (1910) and Warburg (1908) found a similar situation in sea-urchin eggs, in which there was little change in oxygen intake when the oxygen tension varied from double that in air to one-fourth of the same value.

In all of the studies in this last group in which oxygen has actually been measured, the Winkler method has been employed. It is well known that this method, while very satisfactory for the determination of dissolved oxygen in pure water or in salt solutions, becomes untrustworthy when organic material is present in the fluids tested. Heilbrunn (1915) and others have objected to the use of the method in the study of heavy suspensions of protozoa and marine eggs. The presence of iron, found by Warburg (1914) to be contained in sea-urchin eggs in considerable amounts, is known to introduce large errors in the titration. (See Alsterberg, 1926.)

I became interested in this problem after making the observation (1924) that the oxygen consumption of a number of marine invertebrates is directly proportional to the oxygen tension in the sea water, over a considerable part of the normal physiological range. This observation has led me to a reëxamination of the problem in other forms. The present communication deals with some results obtained on unicellular materials in an attempt to confirm the conclusions of previous workers by methods not open to the criticisms which can be leveled against the Winkler technique. This confirmation has been secured. The data are submitted in support of the older observations, and as giving a more complete account of the oxygen tension relationships in the *Arbacia* egg than has previously been published.

On the technical side an attempt has been made to apply standard methods of gas analysis to the study of the problem. Novy and his collaborators have previously successfully used such methods in their study of bacterial respiration. I find that the oxygen consumption of unicellular animal organisms can be similarly followed by such methods, with an accuracy at least as good as that possible in human and mammalian metabolic studies. The carbon dioxide production is more difficult to determine because of the high solubility of the gas in the liquid phase, and the possibility of its chemical fixation. No great reliance can therefore be placed upon the carbon dioxide values given below, or upon the respiratory quotients calculated. The large variations in the value of the quotient is sufficient to indicate the magnitude of the errors which must be present in the determination of carbon dioxide. My main concern has been to study the oxygen consumption.

Experiments with *Paramecium.*

A group of experiments was first carried out with *Paramecium,* in an attempt to develop a satisfactory technique. For several reasons the data obtained are not as complete or accurate as the values secured later on *Arbacia* eggs. The results are, however, fairly consistent and give a satisfactory confirmation of Lund's report on this organism.

A thick suspension of the protozoa was prepared by centrifuging several liters of fluid from a number of cultures. The organisms were then washed through several changes of tap water, being concentrated with the centrifuge after each washing. The suspension in its final form was practically free from bacteria. The cultures were never entirely pure, but *P. caudatum* always constituted at least 95 per cent. of the protozoa present. The presence of other unicellular organisms, either animal or plant, cannot appreciably have modified the results.

A preliminary obstacle was encountered when it was observed that it is exceedingly difficult to secure two samples of such a suspension which will contain the same number of animals. This difficulty arises from the high mobility of the organisms which are negatively geotropic, and tend to rise to the surface even while the sample is being drawn. After many unsuccessful attempts to secure two identical samples, the procedure was abandoned. In its stead it was found possible to carry out two consecutive measurements of respiration upon the same suspension, the first at atmospheric pressure, the second at some lower or higher pressure. Under the conditions of the experiments division was absent, yet the measurements were completed before starvation intervened.

20 cc. of the suspension finally obtained were introduced into a cylindrical glass vessel, of about the size and form of a Haldane gas collecting tube. This tube was fitted with three-way stopcocks at both ends. The volume was 80.85 cc. After the introduction of the suspension the volume of gas in the tube was, therefore,

60.85 cc. Air delivered by a pump under a small pressure was now bubbled through the suspension for five minutes. This air was taken by the pump from a large room in the basement of the medical building; its oxygen content was slightly lower, and its carbon dioxide content slightly higher, than in outside air. The actual percentages were determined by later analysis. At the end of the equilibration period the tube, completely filled with the room air, and with the suspension, in gaseous equilibrium with this air, was closed off, leaving the contained gas completely saturated with water, at atmospheric pressure, and at approximately 25° C., the temperature of the room. The tube was then placed horizontally within a water bath at a temperature of 25° C. ± .2°. From time to time the tube was gently rocked by hand to keep the suspension approximately in gaseous equilibrium with the air above it. At the end of three hours the tube was removed and the suspension vigorously shaken into complete equilibrium with the gaseous phase. A sample of the contained gas was now withdrawn into a Bailey collector, and set aside for later analysis.

As quickly as possible the same suspension was again equilibrated with room air. The tube was then partially exhausted by a water pump, the residual pressure being measured by a mercury manometer connected with one inlet. Upon the attainment of the desired low pressure the stopcocks were closed, and the tube placed again within the water bath. At the conclusion of a second three hour period the gas in the tube was brought to atmospheric pressure and a sample collected. At the end of this second period the organisms were alive and active.

The gas samples were now analyzed by the use of a Haldane-Henderson gas analyser. Whenever possible duplicate or triplicate determinations were made, and the results averaged. Assuming the gaseous solubilities to be those given by the standard tables for pure water at this temperature, the total oxygen and carbon dioxide present at the beginning and at the end, in both air and water, were now calculated, the usual corrections for barometer, water vapor, etc., being applied.

The results obtained in fourteen experiments are given in Table I. It is seen that the oxygen intake is practically constant from 200 to 50 mm. Hg partial pressure of oxygen. Below 50 mm. the values are somewhat reduced, but down to 11 mm. the intake is still at least 80 per cent. of that at atmospheric pressure. Since, in these experiments, an oxygen gradient must have been present from air to water, the actual tensions in the water were somewhat lower than those given in the table, which represent the tensions in the air. The ability of these organisms to utilize oxygen at low tensions therefore becomes even more evident.

TABLE I.

RESPIRATION OF *Paramecium* AT DIFFERENT OXYGEN TENSIONS.

Experiment.	Oxygen Pressure in Second Period.	Respiration in First Period.			Respiration in Second Period.			Ratio between O_2 Consumption in Second Period and that in First Period.
		O_2 Cons.	CO_2 Prod.	R. Q.	O_2 Cons.	CO_2 Prod.	R. Q.	
	mm. Hg.	*c.c.	c.c.		c.c.	c.c.		
1	208–192	1.030	.703	.683	1.027	.753	.733	.997
2	211–195	1.107	.663	.598	1.167	.762	.653	1.054
3	154–139	.969	.565	.583	1.025	.640	.625	1.058
4	154–135	1.345	.849	.632	1.390	.903	.649	1.033
5	122–109	.933	.763	.817	1.029	1.016	.986	1.103
6	92–68	2.088	1.490	.714	2.002	1.446	.722	.952
7	91–74	1.216	1.086	.893	1.245	1.204	.967	1.024
8	70–48	1.654	1.302	.787	1.612	1.448	.898	.975
9	70–60	.698	.390	.559	.724	.458	.633	1.037
10	70–57	1.131	.676	.598	.973	.553	.568	.860
11	60–42	1.645	1.028	.686	1.440	1.008	.699	.875
12	49–28	1.592	1.093	.686	1.546	1.115	.721	.971
13	28–11	1.146	.766	.668	.977	.638	.652	.853
14	28–11	1.642	1.134	.691	1.290	1.038	.804	.786
		Average R. Q.		.685			.736	

* Volume measured at 760 mm. Hg and O° C.

The average of the respiratory quotients obtained in twenty-eight determinations comes out to be .710. Considering the wide range of the individual values it is hardly possible to attach any great significance to this figure, although it may be taken to suggest the presence of a fat metabolism under the conditions of the experiment, when the normal food supply is absent.

These preliminary experiments indicated that the method is applicable to such problems, but certain difficulties were encountered which made it advisable to complete the study on another material.

These consisted in (1) the impossibility of controlling the activity of the organisms, (2) the manipulation of gases at pressures very much below atmospheric, which prevented the exploration of very low oxygen tensions, and (3) the lack of complete gaseous equilibrium between air and water during the course of the experiment. The study was therefore continued with a modified method at Woods Hole on fertilized *Arbacia* eggs, which have no independent motility during the first hours of their development.

Experiments with Fertilized *Arbacia* Eggs.

In these experiments it has been found possible to secure two suspensions of eggs containing equal numbers of cells, whose respiratory exchanges check well with each other when the two are studied simultaneously under identical conditions. The eggs were freed from ovarian debris and body fluid and washed through several changes of sea water. A heavy suspension of cells was secured by permitting the eggs to sediment in a large beaker and then pouring off the greater part of the supernatant sea water. These were then fertilized. About ten minutes after fertilization two 60 cc. samples of this suspension were taken up by pipette and introduced into two tubes similar to that used for *Paramecium* but of a somewhat larger volume.

The lower oxygen tensions were secured by mixing oxygen and nitrogen, or air and nitrogen, in the desired proportions. Eight liters of such a gaseous mixture were collected in a large bottle, over water. One tube (B) was then brought into equilibrium with this mixture, the gas being bubbled through the suspension for at least five minutes. For the same period the second tube (A) was equilibrated with outside air. In every case a sample of gas was collected from the low pressure tube toward the end of the equilibration, and its later analysis accepted as giving the value of the initial oxygen and carbon dioxide percentages. The air which had passed through tube A was analyzed in several experiments and this value accepted for the rest as giving the initial oxygen and carbon dioxide percentages in the high pressure tube. It showed, after passing through the egg suspension, a slight diminution in oxygen and a slight increase in carbon dioxide.

At the conclusion of the equilibration the two tubes were closed in such a manner that the contained gas was left at atmospheric pressure and at approximately 20° C. They were then placed side by side within a water bath, and rotated continually throughout the experiment, turning at the rate of about thirty times a minute. Under these conditions the eggs were always evenly distributed throughout the suspension, and kept in constant motion, the water was always nearly in equilibrium with the gas, and cleavage proceeded in a perfectly normal manner.

Running sea water was used in the water bath. Its temperature varied slightly from day to day. The lowest temperature recorded in any experiment was 18.2° C., the highest 20.2° C. The experiments continued in most cases for two hours; in a few cases for three hours. The first division occurs about one hour after fertilization at this temperature; subsequent divisions follow about every thirty minutes. At the end of the two-hour experiments the eggs were in the four and eight cell stage; at the end of the three-hour experiments they were in the sixteen and thirty-two cell stage. The material is not, therefore, unicellular throughout the whole experiment. The individual cells, however, in all of these early stages are all at the surface of the dividing egg in intimate relation with the oxygen supply in the water; there seems every reason to believe that the relationship under investigation will not be materially modified by this increase in number of cells, unaccompanied by any change in the mass of respiring tissue. We have reason to believe from the work of Gray (1925), that cleavage itself does not affect the rate of oxygen consumption, and that, after the first sharp rise following fertilization the consumption is practically constant during the first three hours of development. The unfertilized egg has so low a gaseous exchange that it has not proven practicable to follow its respiration by the present method.

At the end of the experiment samples of gas were secured from both tubes and analysed. The oxygen and carbon dioxide in the gas and in the sea water were then calculated for the beginning and for the end of the experiment. For this calculation the absorption coefficients for oxygen and carbon dioxide in sea water given in Tabulæ Biologicæ (Vol. 4, pp. 571–578) were used. The results of a typical experiment are as follows:

	Tube A.	Tube B.
Oxygen tensions during experiment	155 to 142 mm. Hg.	61 to 49 mm. Hg.
Volume of tube	106.15 c.c.	105.39 c.c.
Volume of suspension	60 c.c.	60 c.c.
Gas Analysis at beginning:		
O_2	20.87%	8.22%
CO_2	.05%	.02%
N_2	79.08%	91.76%
Gas analysis at end (corrected for volume change):		
O_2	19.16%	6.59%
CO_2	.62%	.58%
N_2	79.08%	91.76%
Oxygen in air and water:		
At beginning	9.961 c.c.	3.863 c.c.
At end	9.147 c.c.	3.095 c.c.
Oxygen Consumption	.814 c.c.	.768 c.c.
Carbon dioxide in air and water:		
At beginning	.045 c.c.	.018 c.c.
At end	.558 c.c.	.517 c.c.
Carbon dioxide production	.513 c.c.	.499 c.c.
Volumes corrected to dry values at 0° C. and 760 mm. Hg.		
Oxygen consumption	.741 c.c.	.699 c.c.
Carbon dioxide production	.467 c.c.	.454 c.c.
Respiratory quotient	.630	.649

Oxygen consumption at low pressure = 94.4% of that at atmospheric pressure.

Carbon dioxide production at low pressure = 97.3% of that at atmospheric pressure.

The results obtained in twenty experiments, carried out after the preliminary tests, are given in Table 2, and shown graphically in Fig. 1. The oxygen consumption is seen to be practically constant from an oxygen pressure of 228 mm. Hg. down to about 20 mm. Hg. Between 80 and 20 mm. there is a definite downward trend in the values, but at 20 mm. the consumption is still about 90 per cent. of that at atmospheric pressures. Below this point the consumption falls off sharply.

In Fig. 1 the experimental values are shown as rectangles. The height of this rectangle corresponds to 1 per cent. on the oxygen consumption scale; the length indicates the oxygen tension range in tube *B* during the course of the experiment. Each rectangle shows that over this range the oxygen consumption of the egg suspension in tube *B* was the indicated percentage of the consumption in tube *A*, run at atmospheric pressure. The absolute

TABLE II.

RESPIRATION OF FERTILIZED *Arbacia* EGGS AT DIFFERENT OXYGEN TENSIONS.

Experiment.	Oxygen Pressure in Tube B.	Respiration in Tube A.			Respiration in Tube B.			Ratio between O_2 Consumption in Tube B and that in Tube A.
		O_2 Cons.	CO_2 Prod.	R. Q.	O_2 Cons.	CO_2 Prod.	R. Q.	
	mm. Hg.	*c.c.	c.c.		c.c.	c.c.		
1	228.8–220.0	.423	.394	.931	.433	.294	.679	1.024
2	155.2–147.2	.473	.430	.909	.470	.386	.821	.994
3	152.2–144.6	.443	.329	.742	.447	.350	.783	1.009
4	142.0–135.7	.309	.257	.832	.315	.237	.753	1.019
5	123.2–112.4	.524	.436	.832	.533	.326	.611	1.017
6	116.8–104.3	.691	.443	.641	.733	.448	.611	1.061
7	85.5– 76.0	.650	.496	.763	.621	.403	.648	.955
8	70.6– 61.7	.572	.417	.729	.520	.412	.792	.909
9	66.6– 55.4	.665	.528	.794	.653	.493	.754	.982
10	61.2– 49.0	.741	.467	.630	.699	.454	.649	.944
11	44.6– 38.3	.390	.334	.856	.370	.339	.915	.949
12	36.8– 24.2	.818	.611	.768	.735	.535	.727	.899
13	30.0– 24.2	.406	.279	.688	.345	.217	.628	.850
14	23.9– 14.9	.592			.524			.885
15	23.7– 8.7	.856	.593	.697	.862	.558	.648	1.007
16	17.3– 10.1	.674	.444	.658	.419	.360	.859	.622
17	11.5– 6.3	.636	.543	.854	.367	.427	1.160	.577
18	7.9– 3.3	.565	.667	1.181	.268	.269	1.004	.457
19	7.1– .8	.746	.527	.706	.309	.488	1.582	.414
20	4.3– 1.7	.665	.445	.669	.151	.222	1.473	.227
		Average R. Q.		.783	Average R. Q. (1–16)			.725

* Volume measured at 760 mm. Hg. and 0° C.

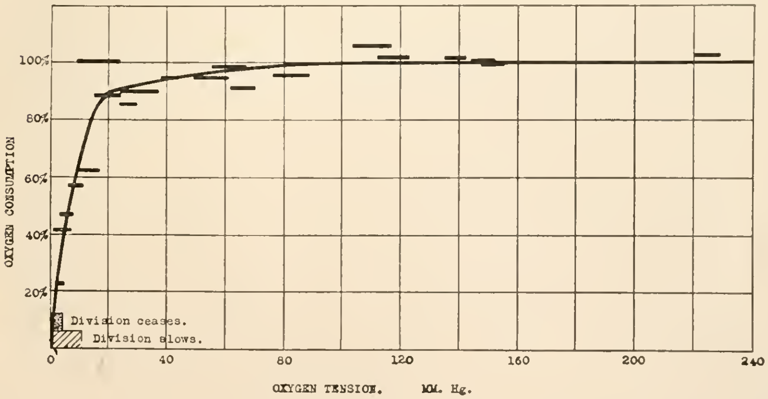

FIG. 1. Oxygen consumption of fertilized *Arbacia* eggs at different oxygen tensions. The range of tensions within which the division rate is affected is also graphically shown.

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values vary considerably from experiment to experiment, but the graph of these percentages assumes a fairly regular and consistent form.

Correlated with the diminished oxygen intake at very low oxygen tensions retardation in development was observed in experiments 17–20. In all other experiments the eggs in the low pressure tube had developed as far as had those at atmospheric pressure. In every case 95–100 per cent. of the eggs developed. In experiment 17, continuing for two hours, a slight retardation in division rate was evident. Counts on 100 eggs from each suspension gave the following values:

	1-cell.	2-cell.	4-cell.	8-cell.
Tube *A* (High O_2).......	4	6	57	33
Tube *B* (Low O_2).......	5	14	74	7

In experiment 18 (2 hours) a more marked effect was observed. Counts on 100 eggs gave the following values:

	1-cell.	2-cell.	4-cell.	8-cell.	16-cell.
Tube *A* (High O_2)........	2	0	52	40	6
Tube *B* (Low O_2).........	54	39	7	0	0

In experiment 19 (3 hours) the eggs at atmospheric pressure were in the sixteen and thirty-two cell stage. In tube *B* about 80 per cent. had reached the four-cell stage, but none were found in later stages. In experiment 20 (2 hours) the eggs at atmospheric pressure were in the four and eight-cell stage. In tube *B* a careful search failed to reveal any cleavage whatsoever. It has long been known that in the complete absence of oxygen cleavage in these eggs is prevented. (See E. B. Harvey, 1926.) My own observations would suggest that a certain minimal concentration of oxygen is necessary for division, but the matter has not received a thorough study. The range of oxygen tensions within which development is either retarded or prevented is indicated graphically in Fig. 1. The values, taken from four experiments, are to be considered as approximations only. Taken in conjunction with the curve of oxygen consumption they show the great ability of these eggs to carry out a normal development down to very low oxygen tensions.

It is of interest to note that in all four of these experiments in which retardation or inhibition of development occurred the respiratory quotient rose above unity; in experiments 19 and 20 the quotient reached the high values of 1.58 and 1.47. These figures suggest the presence of anaerobic respiratory processes at these low oxygen tensions. It is not possible to be certain concerning the matter, since, under these conditions of oxygen lack, acid metabolites may collect in the suspension and liberate carbon dioxide from the carbonates of the sea water.

In none of these experiments has the tension of carbon dioxide risen to such a point that it can have materially affected developmental rate. Haywood (1927) has shown that, in high concentration, carbon dioxide behaves as a narcotic and completely prevents cleavage when its tension rises above 230 mm. Hg. Below this value cleavage occurs at a rate slower than normal. The threshold tension for this carbon dioxide effect to appear was not determined, but it seems evident that at very much lower concentrations the retardation of development must become negligible. The highest carbon dioxide value observed in the present study was at the end of experiment 18, when the partial pressure reached 7 mm. Hg in tube *B*. The retardation of development observed at low oxygen tensions must therefore be caused by oxygen lack rather than by a narcotic effect of the carbon dioxide produced. Haywood also reports experiments on the influence of low oxygen tension upon developmental rate which agree with my own findings in showing practically no influence down to quite low values.

In most experiments carried out below an oxygen tension of 50 mm. Hg there was observed, at the end of the experiment, a liberation of pigment in the suspension in the low pressure tube which became more and more marked as the oxygen tension was lowered. This liberation of pigment apparently arose from the cytolysis of a certain number of cells. The actual percentage of eggs thus destroyed was not determined, but must have been small, since at the end of the experiment the volume of the eggs after sedimentation was not appreciably diminished. The downward trend in the oxygen consumption values below 80 mm. Hg may be in part due to this destruction of a small number of the eggs, although we know, from the work of Warburg (1914) that respiratory exchanges may continue for some hours even in completely fragmented sea-urchin eggs, at a level not far below that found when the cells are intact.

The ability of both protozoa and sea-urchin eggs to carry on a normal respiratory exchange down to very low oxygen tensions points very definitely to the normal presence, within the cells, of a considerable oxygen tension. Oxygen is present in such amount that it does not limit the metabolism, whose rate is determined by other than oxidative reactions.

Summary.

By standard methods of gas analysis the respiratory exchanges of *Paramecium* and of fertilized *Arbacia* eggs have been studied. The respiratory rate in both materials is found to be practically constant over a wide range of oxygen tensions, thus confirming older work done by other methods.

In the fertilized *Arbacia* egg the oxygen consumption is practically constant between 228 and 20 mm. Hg partial pressure of oxygen. Between 80 and 20 mm. Hg there appears to be a slight diminution in oxygen intake, but at 20 mm. Hg the consumption is still about 90 per cent. of that at atmospheric pressure. Below 20 mm. Hg the consumption is sharply reduced.

The cleavage of *Arbacia* eggs proceeds at a normal rate down to very low oxygen tensions. No retardation in development has been observed above 11 mm. Hg. Below this value the rate becomes slower and cleavage ceases entirely below 4 mm. Hg.

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A COMPARISON OF THE OXYGEN CONSUMPTION OF UNFERTILIZED AND FERTILIZED EGGS OF *FUNDULUS HETEROCLITUS*.

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Since Warburg (1) in 1908 measured the oxygen consumption of *Arbacia* eggs and observed the marked increase following fertilization, the oxidation processes in marine eggs and embryos have been carefully investigated. The rate at which the oxygen is removed from the surrounding air or sea water has been correlated with the stages in development. Thus Shearer (2) measured the oxygen consumption during fertilization of Echinoderm eggs, and found a decided increase upon the addition of the sperm; "more oxygen is taken up in the first minute of the process than at any subsequent interval of the same time." In another article Shearer (3) states that, in the first hour of development, the fertilized egg consumes six to seven times as much oxygen as the unfertilized egg. In the star fish egg, however, according to Loeb and Wastenys (4) there is no increase in the oxidation rate after fertilization.

The rate of oxygen consumption is also correlated closely with heat production. Rogers and Cole (5) in their work on *Arbacia* eggs have shown how the heat production varies before, during, and after fertilization; according to them "the rate of heat production at the instant of fertilization is ten to twelve times that of the unfertilized egg."

The literature upon this subject reports work done almost exclusively upon invertebrate eggs. Apparently no previous study of the influence of fertilization upon respiratory rate has been made on any vertebrate egg. Scott and Kellicott (6) and Hyman (7), who have measured the oxygen consumption of *Fundulus* embryos at various stages of development, made no observations on the respiration during the first two hours after fertilization, and secured no information as to the influence of fertilization itself. The present study represents an attempt to secure such information. It has been possible to show by several methods that fertilization markedly increases the oxygen consumption of the eggs of *Fundulus heteroclitus.* The time relations of this increase are of some interest.

1. Winkler Method for Determination of Dissolved Oxygen.

The Winkler method as applied to this problem was employed in the manner described by Amberson, Mayerson and Scott (8). 600 eggs were placed in 500 cc. of sea water in each of two small Erlenmeyer flasks. Samples for analysis were withdrawn through siphons. The water surface was covered with paraffin oil to minimize the diffusion of new oxygen from the air into the water. The sea water was analyzed for dissolved oxygen previous to experimentation; the initial sample was withdrawn after twenty to forty minutes. In order to secure successive determinations of the dissolved oxygen during an extended time, it was necessary to adopt a micro-Winkler method as suggested by Lund (9). For these analyses small vials of 6.5 cc. capacity were used. Fig. 1 represents the graph resulting from plotting the data shown in Table I. below. The values for the amount of oxygen consumed during a given period are obtained by subtracting the amount remaining in the sea water at the end of that period from the amount originally present in the sea water used for the experiment.

Table I.

Time.	Sea Water Originally Contains 5.1 cc. Oxygen per Liter.			
	Unfertilized Eggs.		Fertilized Eggs.	
	O_2 Remaining.	O_2 Consumed.	O_2 Remaining.	O_2 Consumed.
20 min.	5.0	0.1	4.7	0.4
45 min.	4.9	0.2	4.5	0.6
7 hrs.	—	—	3.7	1.4
10 hrs. and 35 min.	4.6	0.5	3.4	1.7
25 hrs. and 25 min.	3.8	1.3	2.8	2.3
31 hrs. and 25 min.	3.1	2.0	1.8	3.3

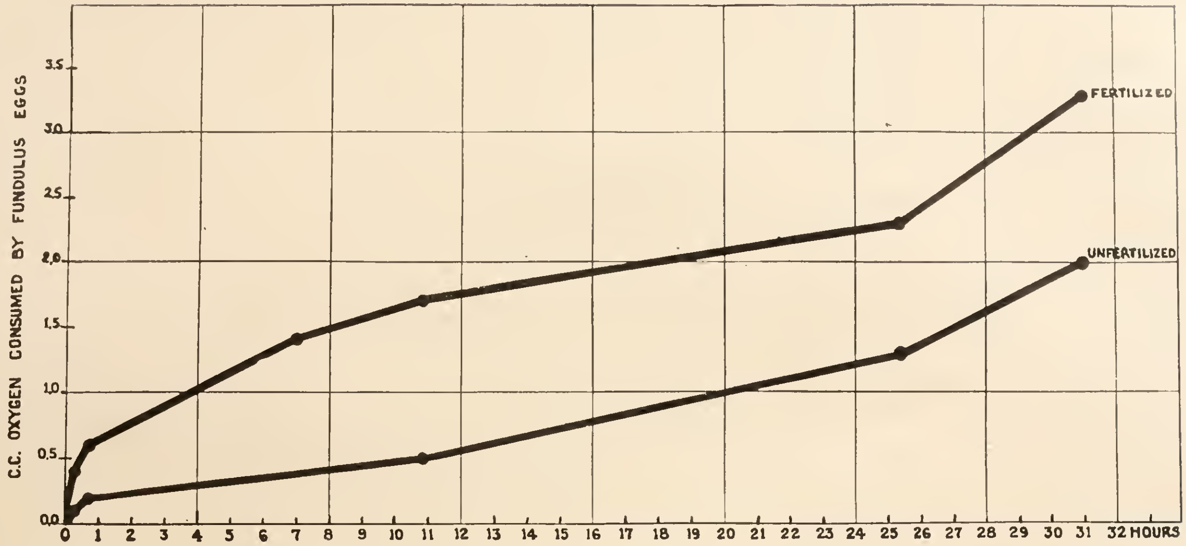

FIG. 1. Results obtained from Winkler Method. The amount of dissolved oxygen found at each successive time is subtracted from 5.1 c.c., the amount of dissolved oxygen originally in the sea water used for the experiment. The total amount of oxygen consumed is thus determined and is plotted against time.

It is to be noticed that the fertilized eggs had at the time of each determination consumed more oxygen than the unfertilized eggs. Data from similar experiments, as shown in Table II., show that the rate of oxygen consumption is most rapid during the first two hours following fertilization.

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Table II.

Time.	Sea Water Originally Contains 5.2 cc. Oxygen per Liter.			
	Unfertilized Eggs.		Fertilized Eggs.	
	O_2 Remaining.	O_2 Consumed.	O_2 Remaining.	O_2 Consumed.
20 min. . . .	5.1	0.1	4.7	0.4
1 hr.	5.0	0.2	4.4	0.8
2 hrs.	4.9	0.3	4.0	1.2
4 hrs.	4.8	0.4	3.9	1.3

2. Micro-Respirometer Method for Determination of Oxygen.

The type of micro-respirometer that was used for the study of oxygen consumption by the *Fundulus* eggs is one that has been devised by W. O. Fenn for similar studies of *Arbacia* eggs. A small glass bottle with a ground glass neck is fitted with a ground glass stopper which is connected with a horizontal fine-bore manometer provided with a centimeter scale. In the center of the bottom of the bottle is a small compartment into which 0.5 cc. of 15 per cent. NaOH is introduced; the eggs to be studied are placed in the space surrounding the compartment. The NaOH serves to absorb the CO_2 produced by the eggs. A small drop of kerosene is introduced into the manometer and its movement across the tube from the outer to the inner end indicates both the amount of oxygen consumed and the rate of the process.

Five cc. of sea water, containing fifty *Fundulus* eggs, were pipetted into the micro-respirometer. Two micro-respirometers were used so that experiments on unfertilized and fertilized eggs could be carried out at the same time under identical conditions. The constants of each apparatus were found by calibration of the respective manometers. The micro-respirometers were immersed in a bath of running sea water; the temperature for all of the

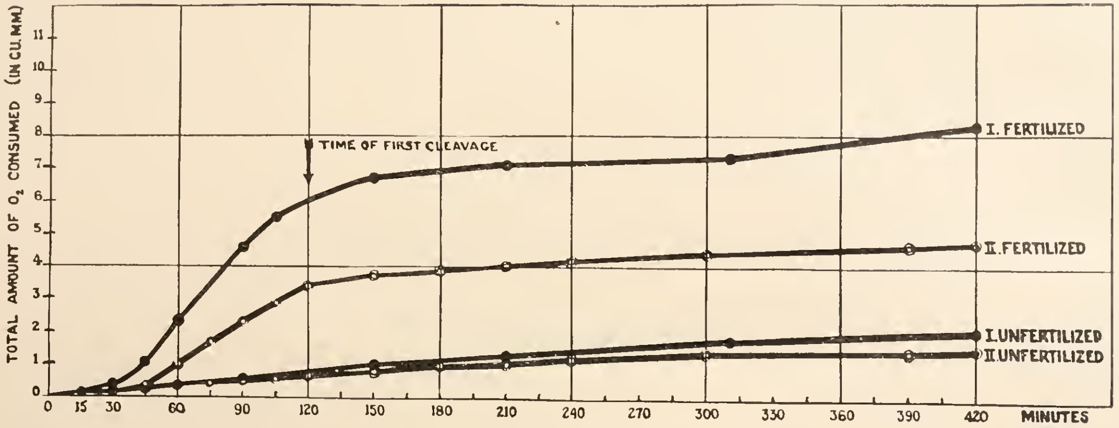

FIG. 2. Results obtained by the micro-respirometer method, showing total amounts of oxygen consumed by fertilized and unfertilized eggs at successive periods. Cubic mm. of oxygen are plotted against time. Experiments I. and II. are chosen to show typical results.

experiments proved to be 20.2 ± .6° C. The readings of the meniscus of the kerosene drop were taken at intervals of fifteen minutes. Over twenty series were run, but in only the last five experiments were the temperature variations observed with a Beckman differential thermometer.

Figure 2 shows typical curves for the results obtained. In Experiment I. the number of cubic millimeters of oxygen consumed by the fertilized eggs is a little less than twice the corresponding amount in the case of the fertilized eggs in Experiment II. Nevertheless both curves show the same marked increase in oxygen consumption 45 minutes after fertilization. This increased oxygen utilization reaches a maximum during the period from 60 to 90 minutes following fertilization. From this time on, the amount of oxygen consumed per unit time falls so that the rate of utilization approximates that for the unfertilized eggs. It would appear, therefore, that the oxygen requirements of the unfertilized *Fundulus* eggs are increased by fertilization. The time relations of this increase are of theoretical interest; they are indicative of some oxidation process occurring within the egg for which an increased oxygen intake is a necessity. To follow the development in relation to the time, two control sets of 50 *Fundulus* eggs were placed in sea water and the stages of development were traced by microscopic inspection. It was found that the increased rate of oxidation occurs at a time before and during the appearance of the groove in the surface of the blastodisc which initiates the first cleavage. The subsequent cleavages evidently do not require such a marked rate of oxygen intake. A single run with twenty 9-day old *Fundulus* embryos revealed a later rise in the oxygen consumption which can be correlated with the marked rise in the rate which Scott and Kellicott found to occur at the time circulation is established.

To show still further the peculiarity of the time relations, the average amount of oxygen consumed per 10 minutes was calculated from the data of Experiment I. and is shown in Fig. 3. The difference in the rates of the fertilized and unfertilized eggs is markedly contrasted.

A few experiments were carried out by a third method and gave results that checked qualitatively with the two mentioned

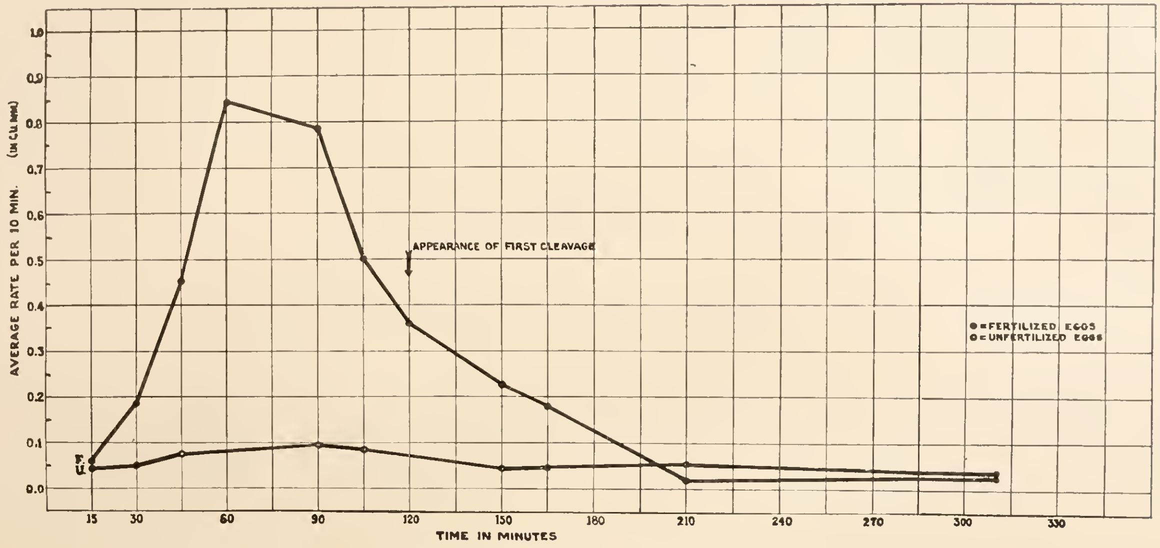

Fig. 3. Respiratory-rate-time graph. The marked increase in the rate of oxygen consumption after fertilization is shown to precede the time of first cleavage. The rate of oxygen consumption of unfertilized eggs is practically constant.

above. This method involved the analysis of air above 25 cc. of water in a 100 cc. Haldane gas collector. At the beginning of the experiment 200 *Fundulus* eggs were pipetted into each of two collectors with the sea water, and the water was equilibrated with atmospheric air which filled the vessel. The collectors were then sealed, immersed, and rotated in a bath of running sea water for two hours. More oxygen was found to have been lost from the sample of air taken from the collector containing the fertilized eggs than from that containing the unfertilized. This method proved to be only approximate as the rotation caused the eggs to stick together in a clump and normal development did not take place. The data secured gave a qualitative confirmation of the more accurate results obtained by the two other methods.

SUMMARY.

By three methods it has been shown that the oxygen consumption of the eggs of *Fundulus heteroclitus* is greatly increased after fertilization. This increased rate of oxygen consumption is at its maximum from 60 to 90 minutes after the addition of the sperm, in a period immediately preceding the first cleavage. The oxygen consumption then falls to a level practically identical with that of the unfertilized eggs.

The writer wishes to express her appreciation to Dr. W. R. Amberson and Dr. W. O. Fenn for their suggestions, and to Mr. J. O. Pinkston for his assistance in the oxygen determinations.

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STUDIES ON *DALLASIA FRONTATA* STOKES.

I. POLYMORPHISM.

GARY N. CALKINS AND RACHEL BOWLING.

Dallasia frontata is a common fresh-water ciliate classified in the family Chiliferidæ, sub-order Trichostomina, order Holotrichida. It was originally described in 1886 by Stokes and characterized by him in 1888 as follows: "Body elongate-obvate, subcylindrical, transparent, longitudinally striate, and finely reticulated, five times as long as broad, the lower or ventral surface convex, the dorsal slightly concave, tapering posteriorly to a somewhat retractile tail-like prolongation forming about one-fifth of the entire body; anterior extremity narrowed, obtusely pointed; oral aperture narrow, ovate, obliquely placed on the ventral or convex surface at some distance from the anterior extremity, enclosing two small vibratile membranes; contractile vacuole single, spherical, near the center of the dorsal or concave border; nucleus presumably represented by large, ovate, sub-central clear space. Length of body 1/180 of an inch. Habitat.—Still water, with *Myriophyllum*" (1888, p. 171).

This characterization is not adequate to describe the many form changes which this remarkable organism passes through in its life history, changes which we are not yet ready to interpret as to cause or full significance, but which we will describe in the following pages.

The organism may be found without much difficulty in the water of Van Cortlandt pond in the environs of New York. Unlike *Uroleptus mobilis* it cannot be regarded as a rare form and is probably widely distributed in fresh-water ponds throughout the country. Many individuals were encountered in the autumn of 1927 and individuals were isolated in different types of media in isolation culture dishes usually employed for this work. Initial experiments with culture media including pond water, hay infusion, and the combination of hay and flour soon showed that the latter, as in the case of *Uroleptus mobilis,* was the most favorable. This medium, made up in the same way as for *Uroleptus mobilis* during eight years of culture, has been consistently used for some of our material since October 6, 1927. Later, media made up with rice and with cracked wheat were tried and some of our material is now successfully running on the latter. In this, as in the hay-flour medium, individuals in the period of maximum vitality divide from four to six times in twenty-four hours.

The material of the isolation cultures is run in " series " and " lines." A series is made up of all the progeny of a single individual isolated as an ex-conjugant; lines, usually five in number, are isolation cultures made from the first five individuals formed by division of the ex-conjugant. Individuals from each line are picked up with a capillary pipette and transferred daily to another culture dish of fresh medium. After such isolations are made the unused individuals of a series are transferred to a Syracuse dish containing about 10 cc. of fresh medium. Such reserve material is allowed to multiply with no change of the medium for from six to ten days. It constitutes a " conjugation test " such as proved successful with *Uroleptus mobilis.* In this way abundance of material is available for study. With Dallasia after a few weeks, epidemics of conjugation occurred in the Syracuse dishes and pedigreed series were started.

Material for preparations has been fixed in osmic fumes, Flemming's, Hermann's and Schaudinn's fluids. The latter, made up as a saturated solution in 95 per cent. alcohol is most satisfactory for general staining. Iron haematoxylin is good for general topography but inner cellular structures are obscured by the dense cortical zone of deeply staining granules. This however, may be avoided by prolonged treatment with turpentine. Auerbach's combination of methyl green and acid fuchsine (without orange G) is excellent for cortical structures and for the mouth parts. Vital stains are useful for demonstrating some structures particularly the capsules about the " couples."

The derived organization of *Dallasia* is so delicately adjusted to its environment that small changes in the latter cause remarkable changes in form. This leads to polymorphism which, more than with any other free-living protozoön known to us, is character-

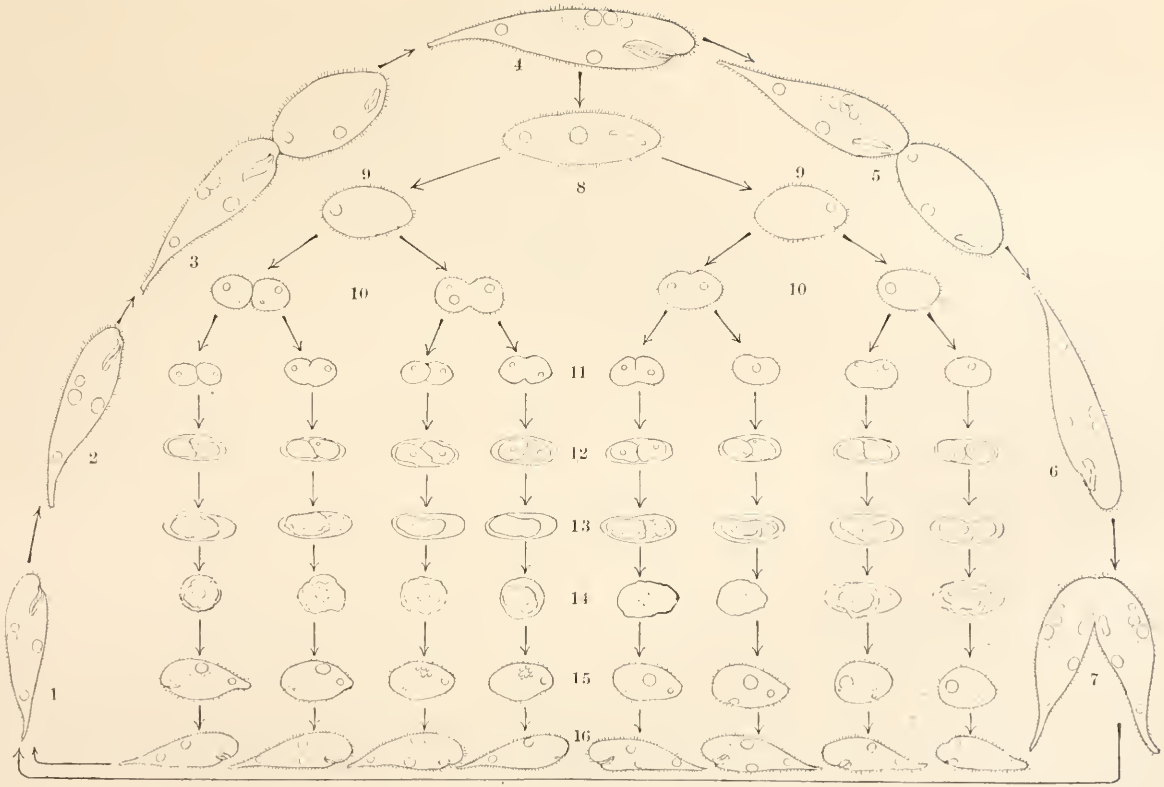

FIG. I. *Dallasia frontata* Stokes. Camera lucida sketches from living organisms. Same magnification throughout. Life cycle. 1, 2. Young tailed forms. 3, 5. Division of tailed form. 6. Tailed form in agglomeration stage, ready for conjugation. 7. Conjugation of tailed forms. 8. Boat type derived from tailed form. 9. First division of boat. 10. Second division of boat without growth, and third division under way. 11, 12. Fourth division of boat forming sixteen individuals which become encapsulated in pairs of sister cells. 13, 14. Fusion of sister cells in capsules, forming eight zygotes. 15, 16. Development of zygotes into small tailed forms.

istic of this ciliate. Certain well-marked types of organization follow in the same order. To these we have applied purely colloquial names which have no resounding classical roots indeed, but which enable us to distinguish clearly between the forms indicated by them. These forms are (1) tails; (2) boats; (3) couples (gametes); and (4) pairs, and they will be described in this order.

1. *Tails.*—This term is an abbreviation for "tail-bearing forms" such as indicated by the original description of Stokes. They are relatively large (105μ to $140\mu \times 22\mu$ to 36μ) and, owing to the remarkable mouth have a curious resemblance to a microscopic shark a resemblance to which Stokes called attention. The anterior end is rounded and in most cases this is the broadest part of the organism which tapers gradually to the posterior end where it narrows into a well-marked tail (Fig. I., 4 and Fig. III., 1). The tail is quite variable in length and shape. Sometimes it is long, resembling the handle of a skillet; again it is reduced until it is little more than the sharply-pointed posterior end of the cell. In other cases the tail disappears entirely. These forms are fairly sluggish, richly stored with food, and are usually attached to the substratum by the tip of the tail where they swing about in circles with the tips of the tails as centers. The cilia are long and closely set in longitudinal rows of which there are about forty.

Another type of tailed form is much longer and somewhat more slender but unlike the fat form is very active and rarely becomes attached.

So far as the visible structures are concerned the most complex part of the organism is the mouth. (Fig. II., 1). This is relatively large and lies in the anterior quarter of the cell. The external aperture varies in shape from an elongated slit to a circular opening. It leads into a spacious buccal pouch (*B. p.*) which extends inwards and diagonally from a region slightly anterior to the mouth, to the gullet which is posterior to the mouth. The entire apparatus is about 27μ long and 15μ wide, thus taking up about one-fifth of the organism. On the floor of the buccal pouch is a long tongue (*T*), triangular in cross section, which runs almost the full length of the pouch (Fig. II.). On one side of this and at the anterior end is a broad endoral membrane which frequently protrudes from the mouth (Fig. II., *E.m.*). At the region of the gullet and on the opposite side of the tongue is a long, narrow undulating membrane, the adoral membrane (Fig. II. *A.m.*). From the base of this membrane to the gullet is a long ladder-like structure recalling the "railroad track" of Chlamydodon (*A.c*). There is evidence of a complicated neuro-motor system which will be described in a later paper on the cytology of these forms.

The macronucleus, like the organism, is polymorphic. In many individuals it appears to be emarginate, frayed out and of a decidedly unhealthy appearance. It is often splinter-like and irregular, but at the approach of division it becomes more condensed and homogeneous and ellipsoidal in form. It divides without mitosis and in the characteristic manner of most macronuclei.

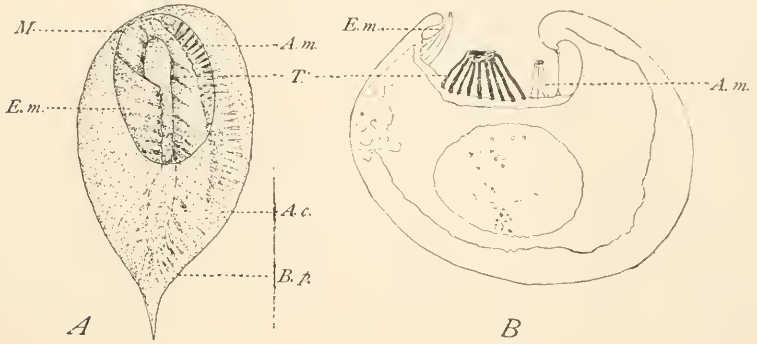

FIG. II. Mouth and buccal pouch of *Dallasia frontata*.
A. Total preparation of tailed form; mouth and buccal pouch only.
B. Transverse section of tailed form.
A.c., ladder; *A.m.*, adoral membrane; *B.p.*, buccal pouch; *E.m.*, endoral membrane; *M.*, mouth opening; *T.*, tongue and supporting bars.

The micronucleus is usually single, spherical, and homogeneous, and is closely applied to the macronucleus. It divides by mitosis.

The contractile vacuole is a single vesicle, in the middle of the ventral surface; feeding canals are absent but a ring of feeding vesicles, clearly visible after contraction of the vacuole, are present.

The cytoplasm is filled with great vacuoles which are frequently so abundant as to distort the organism. They are gastric vacuoles for the most part but are frequently merely fluid-filled vesicles.

Granules of large size and great number are present in all stages of these tailed forms and are a nuisance in preparations stained with iron haematoxylin. The majority of them stain well with the vital dyes neutral red, brilliant cresyl blue and methylene blue.

All in all these tailed forms are remarkably variable in size and shape. They appear to be highly sensitive to environmental stimuli readily becoming amorphous and variously distorted. If the medium is too rich such distortions are more numerous. For some unaccountable reason, possibly because of incomplete reorganization after division, minute dwarf forms with grotesquely large mouths are frequently seen (Fig. III., 4). Such types are prone to change into distinctly amoeboid forms with protoplasmic processes which cannot be distinguished from pseudopodia (Fig. III., 4*a*).

2. *Boats.*—In form and character boats are quite different from the tailed forms. They are considerably smaller (68μ to 83μ) and are derived from the tailed forms by gradual absorption of the attenuated caudal extremity. Both anterior and posterior ends become rounded and the organism becomes navicular and symmetrical (Fig. I., 8; Fig. III., 10). The environmental conditions under which the transition occurs have not yet been fully determined but the period required for it varies according to the age of the series. It is a striking phenomenon to see a rich stock culture in fresh medium yield nothing but boats twenty-four hours later. Such boats are not attached but shoot about the culture dish with amazing speed. After another 24 hours the majority of them have divided four times, giving rise to sixteen minute cells which separate off in pairs to form the couples. After the first division of the boats the daughter cells (gamonts number 1) are more quiet than the original boat and have a tendency to rest on the bottom where the second division takes place. The daughter cells of this second division (gamonts number 2) still have the ability to move but their movement is more or less spasmodic and irregular and their daughter cells (gametocytes) derived from a third division, are now quiescent (Figs. I., 10, and III., 13). These forms, however, are rarely found on the bottom but, together with the couples, are suspended in the medium.

The early stages of the boats show mouth parts only slightly

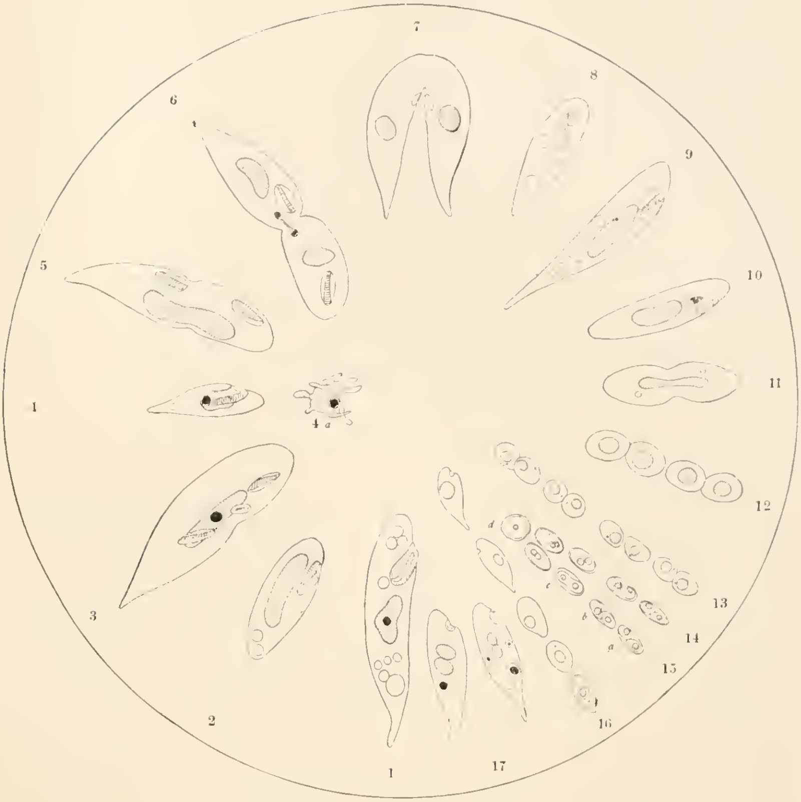

FIG. III. *Dallasia frontata* Stokes. Life cycle. Camera lucida sketches from preparations. Same magnification throughout.

1. Vegetative individual from isolation culture.
2. Boat-shaped individual before tail is formed which may originate at any time from the anterior end of dividing tailed form.
3. Early stage of division of tailed form.
4. Degeneration type of tailed form which may give rise to an amœboid form 4*a*.

5, 6. Later stages of division of tailed form.

7. Conjugation.

8, 9. Ex-conjugants which reorganize into tailed forms.

10. Typical boat-shaped form characteristic of mass cultures after two to three days.
11. First binary division of boat.
12. Second division of boat.
13. Third division of boat giving eight products.

14, 15. Fourth division of boat and fusion of pædogamous gametes in capsules to form eight zygotes.

16. Development of zygote into young individual.
17. Intermediate young individuals from couples.

different from those of the tailed forms but these parts are slowly absorbed and in the second generation of the boats they have entirely disappeared. The macronucleus of the boat form is more condensed, more definite in form, and stains more readily than in the tailed form, and this intensity of staining is retained throughout all of the later stages. The protoplasm likewise is denser and has lost its included vacuoles but there are still many granules which are partitioned out at each division with apparently no increase in their total number. The contractile vacuole varies considerably in position, sometimes on the ventral surface, sometimes on the dorsal and frequently nearer one pole than the other. In the third and fourth divisions of the boats the onset of division is always indicated by the presence of two vacuoles symmetrically placed in the cell.

The first two divisions are fairly slow, requiring several hours but the last two divisions follow one another in quick succession.

Boat-shaped forms may appear at any stage and appear to be a palingenetic phase of the organism. Thus in ordinary division of the tailed form the anterior half is navicular until the tail is regenerated. Also just as pathological tailed forms turn into amœboid cells so the boat-like individuals may undergo a similar pathological change. In some cultures the entire population apparently becomes thus transformed into amœbæ.

3. *Couples.*—The first two divisions of a "boat" lead to small individuals (21μ to 25μ) of broadly ellipsoidal form and with relatively large nuclei (Fig. III., 12). The daughter cells formed by the second division still have the power to move and usually become widely separated. Each divides into two and these two quickly give rise to four. Chains of four cells are characteristic and as there is a tendency at this period for the boats and their products to agglomerate, great masses of these chains are frequently found in the Syracuse dishes. The four cells of a chain soon become associated as two pairs and these are the "couples" (functionally gametes) of our terminology. These pairs measure from 22μ to 26μ, each individual, from 11μ to 13μ. About each couple is a delicate capsular membrane resembling a fertilization membrane, but there is as yet no fertilization, hence the resemblance is closer to a sporocyst membrane of two gregarines in pseudo-conjugation (Fig. I., 13; Fig. III., 14).

We have repeatedly watched the process of couple formation in the living cells and the further changes which take place within the capsule. An instructive picture is obtained by use of neutral red which stains some of the endoplasmic granules and these furnish points of orientation. The two cells of a couple fuse to form a zygote (Figs. I., 13, and III., 15). The nuclei also fuse. We have watched this fusion in living couples under an immersion lens and have noted a center in each gamete where brownian movement of granules is evident. Stained preparations show that these centers are nuclei. After fusion of the cell bodies these centers approach and melt into one immediately after which there is a more violent brownian movement of the granules.

These activities show that the boats are gamonts which give rise to gametocytes and the latter to gametes of which there are sixteen from each gamont. Fertilization is strictly paedogamous and nothing like it has been described for any type of ciliate. The nearest approach to it is Brumpt's account of encystment and fusion in *Balantidium coli,* but here two gamonts come together, no gametes are formed and the two individuals, as hologametes, fuse within a membrane analogous to the sporocyst membrane of gregarines.

This period of copulation is a critical one in the history of *Dallasia.* Up to the present time we have not succeeded in rearing a single zygote in isolation culture. Many young forms are found in the Syracuse dishes in which an epidemic of copulations has occurred (Fig. I., 16); some of these are not yet provided with mouths and their development into mouth-bearing forms has been repeatedly observed (Fig. I., 15). The origin of these young forms from the stage of the encapsulated zygote has also been observed but we have not yet succeeded in providing a suitable environment for their continued life in isolation culture. In many cases, but not in all, the zygote apparently encysts within the capsule (Fig. III., 15*d*) and such cysts are liberated by the dissolution of the capsular membrane. The further fate of these cysts is unknown.

4. *Pairs or Conjugants.*—We have cultivated *Dallasia* in isolation cultures for four months and now have eight series of different ages under observation each series derived from an indi-

vidual ex-conjugant. Pædogamous copulation, described above, occurs in conjugation tests made within a week of the first division of an ex-conjugant and epidemics of such unions still occur at intervals in our oldest series. They occur less frequently and in much milder form when the individuals of a series are mature for conjugation.

Conjugation epidemics are rare. Tests have been made daily by placing the reserve individuals left over after the usual isolations are made, in a Syracuse dish with about 10 cc. of fresh medium. These dishes are set aside in a moist chamber and left for at least one week and usually without the addition of fresh medium. They are examined daily and the observations recorded. In the early life of a series boats usually appear within two or three days and the boats usually give rise to couples. If, however, such boats are transferred to fresh medium they change again into tails. Sometimes fully 100 per cent. of the original tailed forms change into boats and these into couples and zygotes but as a series grows older there is an increasing percentage of tailed forms which do not become transformed into boats and an increasingly diminishing number of couples. In Syracuse dishes with material from older series there is thus a predominance of tailed forms at all stages. These are somewhat smaller (Fig. I., 6) than are the individuals of the isolation cultures and they show the same type of agglomeration as does *Uroleptus mobilis* in similar conjugation tests. As with *Uroleptus* such agglomerations are usually although not invariably, followed by conjugation of the individuals.

The first epidemic of conjugations occurred after thirty-five days of culture of a wild individual and gave us material for Series 2 and *2a* of our pedigreed races. In one of these (*2a*) a mild epidemic occurred in the 96th generation or 25 days after the first division of the original ex-conjugant, and Series 3 and 4 were derived from it. A second epidemic occurred in the 160th generation or 47 days after the first division of the ex-conjugant and from this epidemic Series 5 and 6 were started. Three other epidemics have appeared in Series 3 and 4 and have furnished material for Series 7 and 8.

The conjugating individuals are relatively small (77μ to 102μ) and are always tailed forms. Union occurs as in *Uroleptus* or

Spathidium, etc., at the anterior ends and, again as in *Uroleptus* the mouth parts are not involved. The mouths, however, are greatly reduced and apparently are absorbed, new ones being formed by the ex-conjugants. The period of actual fusion varies from twelve to twenty-four hours and the period of reorganization of the ex-conjugant varies from one to four days. The cytological details have not yet been worked out but meiotic divisions, interchange and fusion of nuclei appear to follow the customary history.

The average division rate for the initial 10-day period is high and is higher in most cases than the division rate for the same calendar period of the parent series. As with *Uroleptus,* however, this is not invariable as the following table shows:

Series 2*a*	division	rate	1st.	10	days,	38.6.	Parent	series	same	period	19.2
Series 3	"	"	"	"	"	36.8.	"	"	"	"	40.2
Series 4	"	"	"	"	"	40.8.	"	"	"	"	40.2
Series 5	"	"	"	"	"	29.6.	"	"	"	"	24.4
Series 6	"	"	"	"	"	33.4.	"	"	"	"	25.2

It is too early to draw any conclusions as regards vitality before and after conjugation, this subject will be discussed in a later study.

Discussion.

So far as we are aware *Dallasia frontata* presents a unique phenomenon hitherto undescribed for the ciliated protozoa. This is the interpolation of a paedogamous fertilization stage in the otherwise ordinary cycle from ex-conjugant to conjugant. Two distinct and entirely different fertilization phenomena in the same life cycle certainly furnish food for reflection, particularly as regards the significance of fertilization in general. The nearest parallel case that we know is *Cryptochilum echini,* as described by Russo. The high death rate, in cultures, after copulation may be significant. It may mean that the culture medium is not suitable for this stage of the organism or it may mean that the encapsulated stage is taken into some other organism where part of the life history is spent as a parasite or as a commensal. Further study of the organisms in culture with experiments to test the effect of different media, which are now under way, may throw more light on this problem.

The novelty of *Dallasia* does not lie in the copulation of micro-gametes; this phenomenon is known in the Opalinidae. Nor does it lie in the union of paedogamous gametes as this phenomenon is well established in the case of *Actinophrys sol* and in the case of *Actinosphaerium eichhornii.* There is certainly no novelty in the phenomenon of conjugation of *Dallasia* for in this it agrees with the vast majority of ciliates. The novelty lies in the combination of fertilization by copulation and fertilization by conjugation.

It is well known through isolation culture work with infusoria that a reorganization process without union of individuals occurs and has the same effect on vitality as does conjugation. It is a process of parthenogenesis termed endomixis by Woodruff and Erdmann (1914); and in some form or other it occurs in practically every ciliate that has been studied. It takes place prior to and during the early phases of encystment in the Hypotrichida, without encystment in various species of *Paramecium.* In *Uroleptus mobilis* endomixis with encystment is a characteristic phenomenon of the early stages of the life cycle (Calkins, 1926); it becomes infrequent with maturity of the protoplasm and is absent altogether in the later stages. In *Dallasia frontata* the incidence of couple formation in the early stages of the life cycle, the formation of capsules, together with the absence of any evidence up to the present, of the ordinary forms of endomixis, lead us to the conclusion that we have here a very unusual, perhaps primitive, type of endomixis. If this conclusion is correct the further hypothesis is permissible that endomixis as ordinarily observed in ciliates is a reminiscence of ancestral gamete-brood formation.

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THE BACTERIOLOGICAL STERILIZATION OF *PARAMECIUM.*

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I.

If for one reason or another it is necessary to control the bacterial content of the medium in which *Paramecium* is living, the first step is a reliable method for the bacteriological sterilization of the animals.

Hargitt and Fray ('17) devised a method which they believed accomplished this end. Their procedure, in brief, consisted in transferring a single animal, by means of sterile pipettes, through five successive washings of sterile water contained in sterile depression slides, the latter being enclosed in Petri plates. There is no evidence in their paper as to how many animals were treated in this way to determine the efficiency of the method. According to Philipps ('22) the technique of Hargitt and Fray " is undoubtedly reliable." However, she used a procedure in her experiments which " made it necessary to wash each animal seven times instead of five."

II.

In his first attempts to sterilize *Paramecia* the present writer increased the number of washings to ten. The animals were obtained from a pedigreed culture of *Paramecium caudatum,* growing on a 0.7 per cent. infusion of pure timothy hay in tap water. This same solution was used for washing. The solution was sterilized in an autoclave at 12 to 15 pounds pressure for 45 minutes.[1]

As a first step eight animals were washed ten times, with the purpose of determining, first, the diminution in the number of bacteria that occurred during the 1st, 3d, 5th, and 7th washes; second, the number of animals sterile in the 10th wash. To accomplish the first purpose, the 1st, 3d, 5th, and 7th wash fluids were plated, these plates incubated at 37.5 degrees C. for 72 hours and examined. For the second purpose the 10th wash fluid together with the animal was broth cultured, and the cultures treated in the same way as the plates. The results are recorded in Table I.

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[1] The difficulty, experienced by Hargitt and Fray, of getting *Paramecia* to live on media sterilized in an autoclave under high pressure, has never been encountered in these experiments though pure lines of *Paramecia* have been carried for a number of months on hay infusions and beef extracts treated in this way.

TABLE I.

REDUCTION IN NUMBER OF BACTERIA IN WASHES, 1, 3, 5, AND 7. EFFICACY OF 10 SUCCESSIVE WASHINGS.

Animal No.	Number of Colonies on Plates of wash Fluid No.				Broth Culture of Wash No. 10 + Animal.
	1	3	5	7	
1	9,000	1	0	0	Infected
2	6,000	3	0	0	Infected
3	10,000	0	0	0	Sterile
4	9,000	2	0	0	Infected
5	13,000	0	0	0	Sterile
6	9,000	0	0	0	Infected
7	16,000	2	0	0	Infected
8	16,000	0	0	0	Infected

The diminution of the number of bacteria in successive washes up through the 5th, as brought out in the above table, concurs very well with the results obtained by Hargitt and Fray. The discrepancy between the number of bacteria present in their first wash and the infection I found may be accounted for by the fact that they transferred only a small portion of these washes to agar plates, while I transferred the entire amount.

Superficially the fact that no animals contaminated the 5th wash might be taken to indicate their sterility. However, 80 per cent. do contaminate the 10th wash. These, of course, could not have been sterile at the time of the 5th washing.

To further test this particular point 18 animals were washed 10 times, and the 10th wash fluid together with the animal broth cultured. The latter was incubated at 37.5 degrees C. for 72 hours and examined. The results are tabulated in Table II.

Of the 26 animals included in these tables only 5 were sterile in the 10th wash.

TABLE II.

EFFICACY OF 10 SUCCESSIVE WASHINGS.

Total Number of Animals Tested.	Broth Cultures of the 10th Wash Fluids + the animals.	
	Sterile.	Infected.
18	3	16

III.

The Hargitt and Fray sterilization method differs from the above method in that they employed a sterile tap water solution for the washing and passed the animals through only 5 wash fluids. Conceivably sterile tap water might be a better sterilizing agent. At any rate, it speeds up animals put into it and leads to rapid reversals which possibly enable the *Paramecia* to throw off more readily the bacteria lodged between their cilia.

Accordingly, 30 animals were washed 5 times in sterile tap water. Broth cultures of the 3d wash fluid and the 5th together with the animal were incubated at 37.5 degrees C. for 72 hours. The results are tabulated in Table III.

TABLE III.

EFFICACY OF 5 SUCCESSIVE WASHINGS.

Total Number of Animals Tested.	Broth Cultures of the 3d Wash Fluids.		Broth Cultures of the 5th Wash Fluids + the Animals.	
	Sterile.	Infected.	Sterile.	Infected.
30	28	2	3	27

Only one conclusion is possible. In the majority of cases 5 and even 10 washings in sterile media cannot be relied upon to sterilize a *Paramecium*.

IV.

As washing is the only practical method for ridding *Paramecia* of bacteria, the following technique was devised.

1. The washings were performed in depression slides, each slide being enclosed in a Petri plate. Those plates in which *Paramecia* were cultured had a thin glass slide under the de-

pression slide, so that the sterile water poured into the plates to make them serve as moist chambers could not get into the depression.

2. The pipettes for transferring the animals through successive washes were made from soft glass tubing having an inner diameter of 2 mm. and a wall of 1 mm. thickness, by drawing this out to capillary fineness. Of the 60 pipettes made in this manner, 10 were chosen at random and the inner diameter measured at the tip of the capillary portion. The average inner diameter was 213 micra; none varied more than 30 micra from this average. The large end was plugged with cotton, and each pipette plugged into a separate test tube.

3. The sterilization of the pipettes and of the depression slides in Petri plates was carried out in a dry oven at between 160 and 170 degrees C. for 45 minutes.

4. The actual washing of the *Paramecia* was performed under a hood which was placed at one end of a large table, and consisted of a wooden frame (3 ft. by 15 in. by 11 in.), with a glass top and cloth sides. The front cloth, which served as entrance, was loose at the bottom. Toward one end there was a binocular microscope with sufficient focal length so that its oculars extended through and above the top. Cloth, with slits in it for the oculars, was glued to the edges of the glass surrounding the oculars.

In handling the animals the transfer pipettes were attached to a rubber tube plugged with cotton and operated by means of mouth suction while the operator was looking through the oculars.

5. All of the various types of culture and wash media used were put into separate, one-liter flasks fitted with glass siphon tubes. Rubber tubing with a glass pipette at one end led off from each siphon tube. After the flasks, tubing and pipettes (the latter plugged into small test tubes) had been sterilized in an autoclave at 12 to 15 pounds pressure for 45 minutes and the corks surrounding the siphon tubes sealed with paraffin, they were arranged outside of the hood and the rubber tubing and pipette led through the back into the hood. The pipettes were suspended at the back of the hood in such a way that their tips, after the test tubes had been removed, did not touch anything. By use of carefully adjusted pinch clamps the size of the drops flowing from

these pipettes was regulated, and hence the volume of media could be determined. The fact that none of the culture media thus treated became infected, although the pipettes were exposed continually for a number of months to the air of the hood, is very good evidence of the efficiency of the hood.

6. The wash fluid was prepared by placing 250 mg. of Liebig's beef into 200 cc. of sterile tap water. This solution was bacterized from the cultures of the pedigreed series of *P. caudatum* being cultured on 0.25 per cent. beef extract, incubated at 37.5 degrees for 2 days and diluted up to 1 liter with tap water. It was placed in one of the liter flasks, sterilized and arranged for use.

7. The actual steps in the washing of an animal were:

(*a*) Three piles of 5 Petri plates each were placed under the hood, and 6 drops (about 1/3 cc.) of wash fluid was put into each of the enclosed depression slides. The lowermost Petri plates served as moist chambers for the 5th wash and hence contained slides under the depression slides.

(*b*) The 15 pipettes necessary for the transfers were placed under the hood.

(*c*) The culture containing the *Paramecia* was placed on the microscope stand and a single individual transferred to the uppermost slide in each stack of plates. Each animal was transferred successively to the depression slide in the Petri plate immediately beneath. By working in rotation from stack to stack, the animals remained in each wash about one minute.

(*d*) When all three animals were in the 5th wash, from 3 to 4 cc. of sterile distilled water was added to the lowermost plates. This prevented excess evaporation from the depression slide while the 5th wash fluid and the animals were being incubated for 5 hours at 25 degrees C.

(*e*) At the end of 5 hours, each animal was again transferred through 4 washes.

(*f*) From the last of these, the 9th, the animal was transferred to the desired culture media. The Petri plate of this, the 10th wash, was converted into a moist chamber as above (*d*).

V.

The data demonstrating the efficiency of this method are summarized in Table IV. In this summary are included the data on those animals which were placed on some type of sterile medium after the 9th wash. The data were obtained by broth culturing the 5th wash after the animal had been in it 5 hours, and the 10th wash together with the animal after the latter had died in it. The death, in some cases, came only after a number of days, during which time the 10th wash and the animal were incubated at 25 degrees C. and examined every 24 hours until the death of the animal. The broth cultures were incubated at 37.5 degrees C. for 72 hours before being examined.

Table IV.

Success of 10 Washings; the Animal Remaining in the 5th Wash Five Hours.

Total Number of Animals Tested.	Broth Cultures of the 5th Wash Fluids.		Broth Cultures of the 10th Wash Fluids + the Animals.	
	Sterile.	Infected.	Sterile.	Infected.
50	17	33	50	0

The number of animals tested and the fact that all were sterile in the 10th wash shows conclusively that the method adopted will rid *Paramecia* of bacteria.

The length of time that an animal is allowed to remain in the 5th wash fluid is a significant factor in accomplishing the sterilization. It raises the question as to why many animals shed bacteria into the 5th wash fluid, but a few do not.

VI.

Tables III. and IV. appear to demonstrate that *Paramecium caudatum* defecates bacterial spores. According to Table III., over 93 per cent. of the *Paramecia* were no longer shedding bacteria into the 3d wash fluid, yet over 90 per cent. of these later proved infected. Table IV. shows that 66 per cent. of the animals left in the 5th wash for 5 hours shed bacteria, yet when these animals were washed four times more they proved sterile.

If the majority of *Paramecia* can be passed through a 3d wash fluid without shedding any more bacteria, and then later do so, it seems highly improbable that the bacteria are on the outside of the animals.

To test this point the following experiments were performed. Seventeen bacteriologically sterile *Paramecia* were left for 24 hours in a pure culture of *Bacillus prodigiosus* in beef extract, while 21 sterile animals were left for the same length of time in a pure culture of *Bacillus subtilis* in beef extract. At the end of this time the animals were washed, individually, ten times; the time of the 5th wash fluid being varied. Those fed on *B. prodigiosus* were allowed to remain in the 5th wash for ½ hour. Those fed on *B. subtilis* were left in the 5th wash from 2 to 5 hours. About 24 hours after the animals had been put into the 10th wash, this together with the animal was transferred to a broth culture, incubated at 37.5 degrees C. for 72 hours and examined. The results are tabulated in Table V.

Table V.

Excretion of Spores by *Paramecia* Fed on *B. subtilis*.

Total Number of Animals Tested.	Bacterial Culture on Which Animal Was Placed.	Time Each Animal Spent in the 5th Wash.	Broth Cultures of the 10th Wash Fluids + the Animals.	
			Sterile.	Infected.
17	*B. prodigiosus*	½ hour	17	0
4	*B. subtilis*	1 hour	1	3
7	*B. subtilis*	2 hours	1	6
4	*B. subtilis*	3 hours	1	3
6	*B. subtilis*	5 hours	6	0

Bacillus prodigiosus has never been known to produce endospores, while *Bacillus subtilis* produces endospores very readily.

In every case the *Paramecia* that had been cultured in *B. prodigiosus* were able to throw off all bacteria, although the animals remained in the 5th wash fluid only ½ hour. Eighty per cent. of the *Paramecia* cultured on *B. subtilis* and then left in the 5th wash fluid up to 3 hours were not sterile when washed five times more.

Since both of these species of bacteria have the same type of flagellae, it seems very unlikely that *B. subtilis* could remain lodged between the cilia of the *Paramecia* for a longer period of time than *B. prodigiosus.*

The above data seem to clearly indicate that *Paramecium* defecates solid material, in this case bacterial spores.

The efficiency of the sterilization technique adopted is further attested by the data in Table V. Those animals washed after having been cultured in *B. subtilis,* and left in the 5th wash for 5 hours, were all sterile in the 10th wash.

The author wishes to thank Professor Otto C. Glaser for aid rendered in the preparation of this manuscript.

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THE EFFECT OF MATERNAL AGE AND OF TEMPERATURE CHANGE IN SECONDARY NON-DISJUNCTION.

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Introduction.

The following figures present the results of the raising of XXY Drosophila females in an effort to ascertain, particularly, how increasing maternal age and how differences in the temperature, at which mother flies are maintained, affect the percentage of exceptions to sex linkage.

The first part of the paper deals with the effect of maternal age and here, because of some lack of conformity in the results obtained at different times, the problem has not been solved. However, I feel that the figures should be published for they have been accumulating for three years and I am not, at present, continuing the investigation. The second part of the paper deals with the effect of differences in temperature. Here the different tests which were made check in a fairly satisfactory manner. I am also able to give some figures, in the last part of the paper, which have a bearing upon the genetic variation in exception-producing ability.

Effect of Maternal Age.

The first experiment was carried out during the time I was a graduate student in Genetics at the University of California, the use of successive subcultures being a routine method of rearing flies there and fermented banana the food medium. I undertook, under the direction of the genetic staff, a rather complete repetition of Bridges' 1916 experiments and obtained comparable results in most particulars. However, in a group of females continuously outcrossed and presumably producing a "normal" percentage of exceptions, I obtained 3423 (5.90 per cent.) of these in 58037 flies; this being somewhat higher than the 4.3 per cent. reported by Bridges.

An inspection of my material, made after most of the data were in, showed that a given female's later subcultures produced, during most of her lifetime, almost one per cent. more exceptions per week than her earlier subcultures. The total production of offspring and of exceptions to sex linkage (exc.) of 109 white-eyed females mated with red-eyed males, and changed each week to a new subculture, is given in the totals column of Table I. The curve of these percentages is that of group 1, Fig. 1.

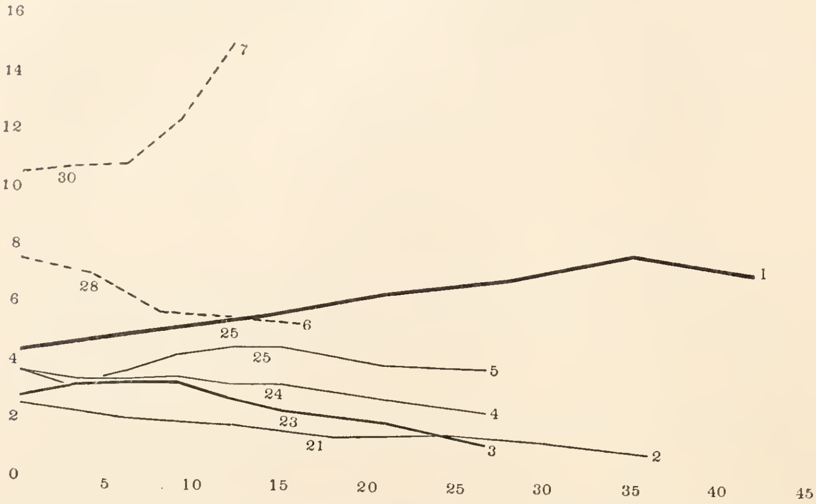

Fig. 1. Curves of smoothed percentages of exceptional young obtained from groups of females kept at different temperatures. Abscissas approximate the age in days of mother females when eggs were ripened. Ordinates are percentages of exceptional young. Curves end with the group number. Under each curve is the temperature in degrees C. Broken lines denote inbred groups. Thick-lined curves are based on more flies.

In order to eliminate the possibility of the weighting of the later subcultures by reason of the longer life, or the greater production of exceptions in certain subcultures of a few high-producing females, I excluded, in the figures presented in Table I., the data from all females which produced over 10 per cent. of exceptions or which had any sub-culture failure prior to the one which ended the females production. When the material is divided into groups of females which produced young for the same length of time,

Table I.

The Number of Young and the Number and Percentage of Exceptions to Sex Linkage in Group 1 when Subdivided into Cultures Produced by Females Living an Equal Length of Time.

Subcultures 7 days apart.

	Subculture													
	A		B		C		D		E		F		G	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	838	33												
% exc.		3.9												
	1,541	78	1,246	66										
% exc.		5.1		5.3										
	2,304	93	2,356	84	2,254	131								
% exc.		4.0		3.6		5.8								
Smoothed %		3.9		4.5		5.0								
	2,003	88	2,319	121	2,111	146	1,781	119						
% exc.		4.4		5.2		6.9		6.7						
Smoothed %		4.7		5.5		6.2		6.8						
	1,840	68	1,790	96	1,571	108	1,845	114	1,150	100				
% exc.		3.7		5.4		6.9		6.2		8.7				
Smoothed %		4.2		5.2		6.1		7.1		7.6				
	1,519	53	1,376	81	1,246	60	1,229	68	1,310	88	782	71		
% exc.		3.5		5.9		4.8		5.5		6.7		9.0		
Smoothed %		4.2		4.7		5.4		5.7		6.8		8.0		
	1,087	53	859	39	682	43	1,091	53	782	66	838	70	475	21
% exc.		4.9		4.5		6.3		4.9		8.4		8.4		4.4
Smoothed %		4.8		5.1		5.1		6.3		7.0		7.5		6.8
Total	11,132	466	9,946	487	7,864	488	5,946	354	3,242	254	1,620	141	475	21
% exc.		4.2		4.9		6.2		6.0		7.8		8.7		4.4
Smoothed %		4.4		5.0		5.6		6.4		6.9		7.8		7.1

the same sort of " curve of age " is obtained. The production of groups of females, so arranged, being also presented in Table I.

All the females used, in obtaining the above figures, descended from the one original white-eyed female which started the XXY line, so the X chromosomes were presumably constant throughout. Male flies, however, were taken from a number of stocks of flies trapped around Berkeley, or present in the laboratory, so the Y introduced into each generation was not constant. The flies were kept in a large cabinet incubator at 25 degrees C. and counted daily. It is perhaps worth recording that the mother female, of each group of subcultures, was removed from the incubator in the subculture tray during the counting period. This was long enough, at times, to cool the vials down to room temperature.

Before leaving an account of this part of the work I should like to report briefly upon a test of the constitution of 60 regular daughters of XXY females. Twenty-seven of these daughters, when mated with Bar males, produced no exceptions. Thirty-three produced exceptions, but of this number 8 daughters produced just one or two exceptions in large counts of flies, the percentage being, in these cases, consistently in the neighborhood of one third of one per cent.; the total production being 10 exceptions (9♀ 1♂) in 3,148 flies. If these 8 regular daughters were XX females which produced primary exceptions these latter are not predominantly male as in Safir's results (1920).

Later Experiments.

After some lapse of time during which I was engaged in breeding Peromyscus I returned to the problem of non-disjunction with the idea of checking my results prior to publication, and also of trying out the effect of temperature differences upon the percentage of exceptional offspring. I obtained a stock of flies, through the courtesy of Dr. R. E. Clausen, and after inspecting the progeny of white-eyed females, mated with normal males, picked up an exception-producing strain. The flies used in these latter experiments consisted of a number of white-eyed females from this stock, a number of white-apricot compound females, obtained from a mating of white and apricot, and finally of a number of white and apricot females obtained by equational non-disjunction from the white-apricot stock, females of which produced XXY daughters pure both for white and for apricot. I could not observe that these allelomorphs, white and apricot, differed from one another in the capacity for exceptional production, in comparable experiments.

The culture methods in this latter part of the work were modified somewhat. Yeast-seeded banana agar was used for food. Females which were producing young were left continuously in the incubator except during the interval when they were changed to a new culture. Subcultures were made up every six days, at 20 or 21 degrees C., and every three days (in group 6, Table III., every four days) at temperatures higher than this. Two Freis electric incubators and one electrically controlled cabinet incubator was used and the temperature checked daily. These machines will fluctuate in temperature, within a degree up or down, but since the routine involved the growth of flies, at each different temperature, over a considerable period of time such fluctuations should cancel out.

Table II. summarizes and Fig. 1 depicts graphically the results obtained when groups of females were kept continuously at certain temperatures each female being transferred to new subculture vials as long as she remained fertile. Except in groups 6 and 7, in which inbreeding was the rule, male parents came from several cultures. In group 6 one inbred stock of wild males was used, in group 7 exceptional brothers.

A comparison of Tables I. and II. and of the curves in Fig. 1 brings to light some very obvious differences in the characteristics of the females in comparable groups. In group 1 the average fertile lifetime, in round numbers, was 28 days. In groups 4 and 5, kept at comparable temperatures, the average fertile lifetime was 16 days. It appears improbable that this difference is an environmental one for banana agar is generally conceded to be a better food medium than fermented banana.

The percentage of exceptional progeny produced by the females in groups 4 and 5 (3.14 and 3.88 per cent.) is considerably below the 5.49 per cent. of exceptions obtained in group 1, and this is not due to the difference in longevity for the group 1 percentage is higher at any comparable age.

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Table II.

The Number of Young and the Number and Percentage of Exceptions to Sex-linkage Obtained from Groups of XXY Females Kept at Different Temperatures.

Gr.	Temp.	Subcultures																	
		A		*B*		*C*		*D*		*E*		*F*		*GH*		I etc.		Total.	
		No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.	No.	Exc.
2.	21°	330	6	571	19	915	11	984	15	740	11	563	9	354	1	—	—	4,457	72
	%		1.8		3.3		1.2		1.5		1.5		1.6		0.3				1.62±.19
	Sm. %		2.5		2.0		1.8		1.4		1.5		1.3		0.9				
3.	23°	3.188	90	2,880	83	2,785	101	1,930	58	1,530	40	1,092	25	658	9	113	1	14176	407
	%		2.8		2.9		3.6		3.0		2.6		2.3		1.4		0.9		2.87±.14
	Sm. %		2.8		3.1		3.2		3.2		2.7		2.3		1.9		1.2		
4.	24°	935	38	1,301	37	954	31	1,156	43	1,131	35	998	28	1,193	42	727	10	8,395	264
	%		4.1		2.8		3.2		3.7		3.1		2.8		3.5		1.4		3.14±.19
	Sm. %		3.6		3.3		3.3		3.4		3.2		3.2		2.7		2.3		
5.	25°	610	25	966	28	844	23	863	44	805	38	579	19	856	44	502	13	6,025	234
	%		4.1		2.9		2.7		5.1		4.7		3.3		5.1		2.6		3.88±.25
	Sm. %		3.6		3.1		3.6		4.2		4.5		4.5		3.9		3.8		
6.	28°	1,239	100	976	60	726	45	639	29	277	17							3,857	251
	%		8.1		6.1		6.2		4.5		6.0								6.51±.40
	Sm. %		7.5		7.0		5.7		5.5		5.3								
7.	30°	1,213	129	740	76	566	64	328	37	127	25							2,974	331
	%		10.6		10.3		11.3		11.3		19.7								11.13±.58
	Sm. %		10.5		10.7		10.8		12.3		14.9								

The curve of age, so obvious in group 1, is not exactly reproduced in any of the later groups although a curve of age, as may be noted in Fig. 1, is usually apparent instead of a randum fluctuation from a model percentage. This curve of age differs at different temperatures, in the figures presented, but the possibility that genetic differences in the females used may be responsible for this cannot be excluded.

A noticeable thing in Table II. is the increase in exception production at higher temperatures. Based on the totals in each group the increase was slow, for a given temperature increment, up to 25 degrees, then more rapid between 25 and 28 degrees and most rapid between 28 and 30 degrees. For these temperatures the curve of increased exception production is comparable to the curve of increased crossing-over of second chromosome genes in Plough's results.

Effect of Temperature Change During the Lifetime of XXY Females.

In order to make tests of this kind females were hatched, and their first eggs ripened at a high temperature and then kept for the remainder of their lifetime at a temperature ten degrees lower. In addition, females were hatched at a relatively high temperature and, after a period at a temperature eight degrees lower, they were returned to the high temperature again. A difference of eight degrees was substituted for ten because too many females died when transferred from a 20 to a 30 degree incubator.

It was found by Plough and Metz that females about twelve hours old have approximately 140 oöcytes in their ovaries. The practice in the experiments here recorded being to empty cultures every 24 hours it can be assumed that the average age at mating, of mother females, was not greater than twelve hours, for younger-appearing females were always chosen for mothers since they are most likely to be virgins. It takes such females six to eight days (this period must differ at different temperatures) to lay the eggs ripened at the temperature at which they were hatched, the lag of layed after ripened eggs continuing all through a female's lifetime.

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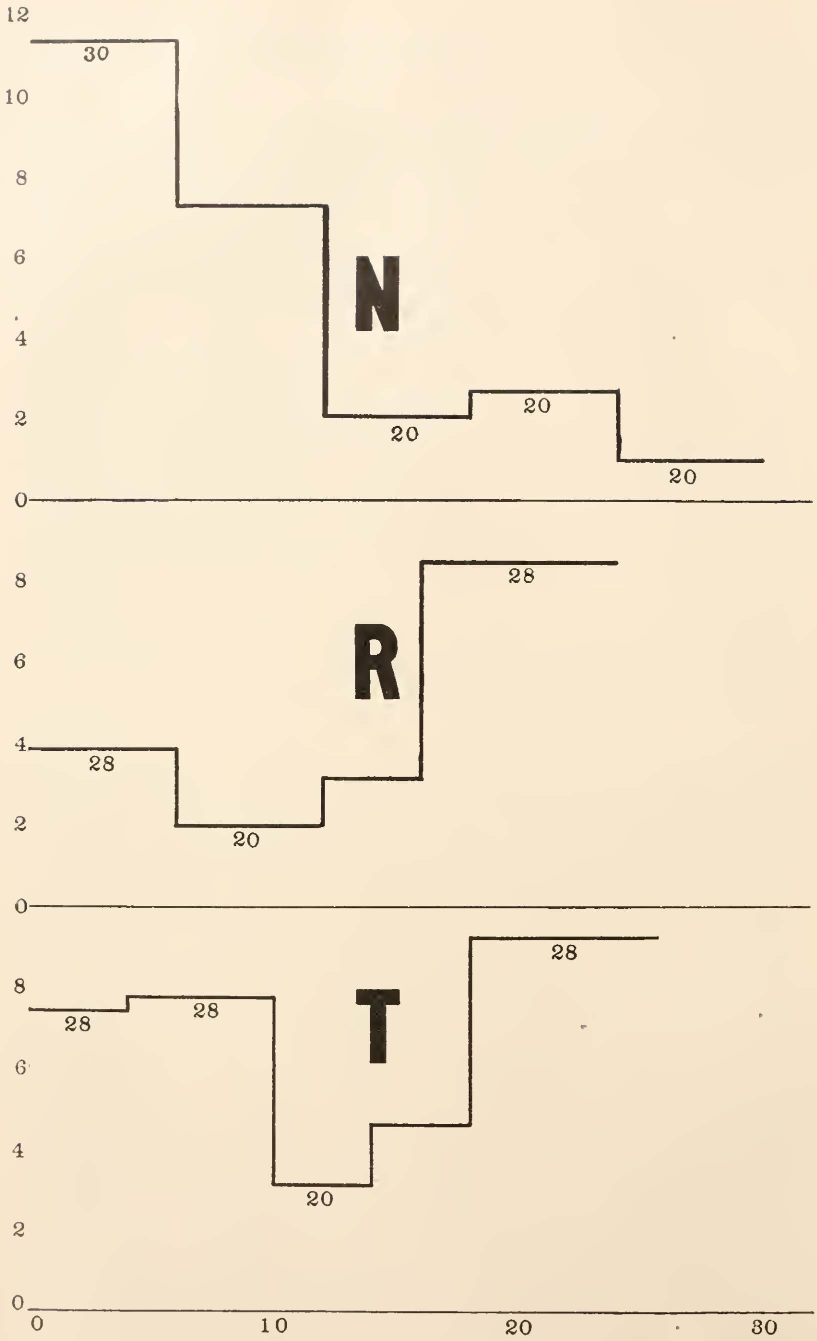

FIG. 2. Curves of three tests of temperature change. Abscissas are days of life of mother females, ordinates the percentages of exceptional young. Figures at intervals, under the curves, are temperatures at which the exception-producing eggs in mother females presumably ripened, intermediate temperature plateaus being left blank.

Table III. and curve *N*, Fig. 2, presents the results obtained when white-apricot compound females, born at 30 degrees C., were mated with normal males and kept at 20 degrees C. Subcultures are six days apart.

TABLE III.

THE NUMBER OF FLIES AND THE NUMBER AND PERCENTAGES OF EXCEPTIONS PRODUCED BY XXY FEMALES BORN AT 30° C. AND THEN MATED AND KEPT AT 20° C.

Temperatures are those at which the eggs, producing the exceptions, are presumed to have ripened.

Subcult.	Temperature.									
	30		30–20		20		20		20	
	A		*B*		*C*		*D*		*E, F*	
	Flies	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	1,751	197	2,005	146	1,931	40	1,023	28	482	5
%		11.2		7.3		2.1		2.7		1.0

It would be expected that the eggs ripened at 30 degrees would be laid in the *A* and in the first part of the *B* subcultures, being followed by eggs ripened at 20 degrees, which would be laid in the latter half of the *B* and in subsequent subcultures. If the percentage of exceptions varies with the temperature at which the eggs are ripened then the *A* subculture should contain the highest, *B* an intermediate and all subsequent subcultures a low percentage of exceptions. It may be seen that this expectation is realized completely.

Table IV. and curve *R*, Fig. 2, presents the results obtained when white-apricot females were born at 28 degrees C., mated with normal males and held for two subcultures (12 days) at 20 degrees C., then returned to 28 degrees C. again.

The expectation of a high *A*, intermediate *B*, low *C*, intermediate *D* and of high subcultures subsequent to *D*, is only partly met. The poor fit in the *A* and *B* subcultures may very well be due to the chance selection of very young mother females. The final cultures are higher than expected.

Table IV.

The Number of Young and the Number and Percentages of Exceptions Produced by XXY Females Born at 28° C. Mated and Held for Two Subcultures (12 Days) at 20° C. and then Returned to 28° C.

Temperatures are those at which eggs are presumed to have ripened.

Subcult.	Temperature.									
	28		28–20		20		20–28		28	
	A		*B*		*C*		*D*		*E, F*	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	1,084	42	1,146	23	834	15	562	18	434	37
%		3.9		2.0		1.8		3.2		8.5

Table V. and curve *T*, Fig. 2, presents the results obtained when apricot females were born, mated with one inbred stock of wild-type males and held for one subculture (4 days) at 28 degrees C., then kept in a subculture (6 days) at 20 degrees C. and finally returned to 28 degrees C. for all subsequent four-day subcultures.

Table V.

The Number and Percentage of Exceptional Young Produced by XXY Females Born, Mated, and Kept 4 Days at 28° C., then Kept Six Days at 20° C. and then Returned to 28° C.

Temperatures are those at which eggs are presumed to have matured.

Subcult.	Temperature.									
	28		28		20		20—28		28	
	A		*B*		*C*		*D*		*E, F*	
	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.	Flies.	Exc.
	2,342	175	3,825	297	2,108	67	1,033	49	485	45
%		7.5		7.8		3.2		4.7		9.3

This was the most adequate test of the effect of temperature for a fair number of flies was raised, the males used as parents were all of one type, and other females (group 6, Table III.) were held continuously at 28 degrees. The *C* subculture should be the low one but should not go down to 2 per cent. for the 6 days at 20 degrees would hardly complete the oviposition of all the eggs brought into the culture which were ripened at 28 degrees. The final cultures are again too high and the same cause may be operative here as in Table IV. I suggest an over-reaction to heat after a transfer from a low temperature.

When it is considered that the curve produced by the percentage of exceptional young in different subcultures, which received eggs ripened at different temperatures, is superimposed upon a curve of age which has been shown to be somewhat variable, it may be concluded that the above figures are as close to the requirements for a demonstration of the effect of temperature as could be expected. I think the statement that the percentage of exceptional progeny, produced by XXY females, varies in being higher at high temperatures and lower at low temperatures between 20 and 30 degrees C. is thus quite warranted by the results.

Genetic Differences in Exception Production.

By breeding to reintroduce the maternal Y chromosome, Bridges obtained a " high " line of exception producing females. He suggested that this high production was due to increased heterosynapsis resulting from the peculiar constitution of the introduced Y. Bonnier, using Bridges' high line, showed that the introduction of a new Y is not followed by a return to the " low " percentage of exceptions, and presented evidences to show that a high percentage of exceptions is a matter of the constitution (gene basis) of the two X's.

The following data suggest that neither of these theories will account for all the inherent differences in exception producing ability. I mated 24 matroclinous daughters of a white-eyed XXY female (♀ No. 116), mated with one male, which had herself produced 14.5 per cent. of exceptions in 447 young. These daughters (they are included in Table I. above) produced an average of 6.2 per cent. of exceptions in 11,692 young, the percentage of individual cultures being from 2.9 per cent. in one culture of 820 young to 10 per cent. in another culture of 600 young. This difference between 2.9 and 10 per cent., is five times its probable error[1] although these daughters all had, theoretically at least, equivalent sex chromosomes. Here an apparent segregation of exception-producing ability is manifest.

It can readily be demonstrated in another way that this variability is not just a matter of random sampling, for if the percentage of exceptions produced during the first part of a female's lifetime is compared with that produced during a later part of her lifetime, positive correlation may be observed. I made such calculations within groups of females which had produced a sequence of complete subcultures at 23, 26 and 30 degrees (groups 3, 1, and 5 already tabulated) and obtained the following correlation coefficients: + .56 ± .12, + .39 ± .07 and + .70 ± .11. This suggests that the ability to produce a certain percentage of exceptions, tends, like any other measurable character, to stay within the limits proscribed by genetic constitution of the individual in question. That being the case one would expect parent-offspring correlation, in exception-producing ability, of about the same magnitude as that which has been found for other characters. I had one series in which I could test this point, for in group 1 there were 60 daughters which had produced 300 or more young which had mothers with an equal productivity. The mother-daughter correlation in exception production was here + .37 ± .07.

I selected a few generations of flies for increased exception-production by choosing the daughters of my higher producing females and mating these daughters with their exceptional brothers. In the first selected generation I obtained 6.5 per cent. of exceptions in 4,493 flies, in the second generation 8.4 per cent. of exceptions in 4,414 flies, and in the third generation 14.6 per cent. of exceptions in 1,772 flies. Exceptional females from average exception-producing mothers which had been similarly mated with their exceptional brothers produced 5.6 per cent. of exceptions in 5,674 flies, so inbreeding alone did not increase the per cent. of exceptions. An inspection of the pedigrees showed the selected high line to altogether come from one high-producing female. After one generation produced by outcrossing to non-related stock, two females from this line still produced 14.1 per cent. of exceptions in 546 flies.

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[1] When the probable errors are obtained by the formula $\pm\sqrt{\frac{p\cdot q}{N}}$, where p = the percentage, $q = 100 - p$ and N = the number of observations upon which p is based.

I also repeated Bridges' scheme of mating to reintroduce the maternal Y chromosome from a high-producing female by mating her daughters with their exceptional uncles. In two generations of this line I obtained 15.5 per cent. of exceptions in 2,072 flies, a figure comparable, since sister females were used in the two cases, to the third and one later generation of the high selection tabulated above. Either of these methods of inbreeding would tend to concentrate factors favorable to exception production in a given line of flies and these need not be intrinsic characteristics of the sex chromosomes.

Conclusions.

The data presented above together with those of previous investigations show that the percentage of exceptions to sex linkage may be affected by a number of variables which, in order of importance, are: the genetic constitution of the female, the temperature at which eggs are ripened, and maternal age.

With regard to genetic constitution, XX females produce well under 1 per cent. of primary exceptions. XXY females may produce from 1 to over 20 per cent. Our knowledge of the causes of this latter variability does not appear to be complete. Bonnier's outcrossing experiments appear to remove the probability that the constitution of the Y chromosome is responsible for Bridges' high eosin line and although Bonnier was not able to exclude the possibility that autosomal genes were implicated, his experiments pointed to an exclusively X chromosomal effect in the production of different percentages of exceptions. It is the rule to have a constant pair of X chromosomes in all lines ot secondary non-disjunction, except as the X's may interchange material with each other or with the Y, and yet genetic variability is still present. The only inference that is possible is that the percentage of exceptions may be affected directly by interaction of the sex chromosomes themselves and indirectly by autosomal genes.[2] This is what has been found to be true in cross-over percentages.

The direct effect of temperature upon the percentage of exceptions appeared in all the tests I made. The results of temperature differences upon protoplasm in modifying physiological activity are so well known that some temperature effect would be an à priori expectation in secondary non-disjunction. Although my data suggest that the temperature effect increased as 30 degrees C. was approached, I cannot exclude the possibility that this was due to genetic differences in the groups of females kept at these different temperatures.

Maternal age appears to affect the percentage of exceptional young to some degree but apparently by interacting with other variables for different age curves were found in different groups and at different temperatures.

The inference that autosomal genes, temperature and maternal age all affect the allocation of the sex chromosomes, in XXY females, to gamete-forming cells, follows the conclusions reached above.

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[2] This would explain why a high-producing female may or may not establish a high exception-producing line.

OXYGEN CONSUMPTION OF INSECT EGGS.[1]

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Iowa State College.

Although insect eggs present unique material for studies in metabolism the literature contains comparatively few reports in this interesting field of insect physiology. Bodine (1) has presented data from a study of eggs of Orthoptera which show the velocity of development to increase in direct proportion to increase in temperature within the normal limits of development. This is in accordance with other findings on the effects of temperature on poikilothermos species. This author shows that it is possible to calculate the time of hatching of eggs if previous temperature history is known. Such knowledge of insect pests may lend itself to practical application.

Fink (2) has conducted studies which lead him to conclude that the formative period in the development of eggs of certain insects is dependent upon whether they are deposited upon foliage or in the soil. Data to be presented in this paper tend to disprove the above explanation. For further references to literature in this field see the papers of Bodine and Fink cited above.

Thanks are due Doctor Erma Smith, Professor of Physiology, and other members of the Zoölogy and Entomology staff at Iowa State College, for many helpful suggestions and encouragement throughout the course of this work.

Purpose.

The purpose of this paper is to present briefly a preliminary report of a study of the oxygen consumption during embryonic development of certain insects.

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[1] Contribution from the Department of Zoölogy and Entomology, Iowa State College, Ames, Iowa.

METHODS AND MATERIAL.

Bodine's modification of Krogh's manometer was used for determining the oxygen intake. Constant temperatures were maintained by use of a Freas electric water bath. The data presented were determined from the eggs of the following insects: Squash bug, *Anasa tristis* De G.; Luna moth, *Tropæa luna* L.; Cecropia moth, *Samia cecropia* L.; and Smartweed borer, *Pyrausta ainslici* Hein.

The O_2 consumption is expressed in millograms of O_2 per gram live weight (exclusive of shell) per hour. Apparently previous workers have not taken into consideration the weight of the shell. If the weight of the shell be deducted, as it evidently should be, the O_2 consumption curve will be raised from 10 to 30 per cent.

The per cent. of shell at the beginning of incubation for several species of insects was found to be as follows: *A. tristis,* 29.2; *S. cecropia,* 22; *T. luna,* 23.3; and *P. ainslici,* 31.

Assuming the weight of the egg shell to remain constant throughout the incubation period, the percentage of shell varies directly with changes in the weight of the egg. It is thus evident that changes in the weight of the egg will alter the type of curve representing O_2 consumption. For this reason the weight was determined just prior to each gas determination and calculations made accordingly.

Determinations were made on egg masses as soon as they were deposited and every 12 or 24 hours thereafter, depending on the length of the incubation period, until hatched. Calculations were made according to the formula of Krogh (3).

TEMPERATURE.

The effects of temperature upon biological processes are too numerous and too well known to warrant detailed discussion. Numerous investigators have studied the effect of temperature upon the length of the incubation period, but few reports have been found dealing with the effect of temperature upon the rate of metabolism as determined by the oxygen consumption. With this in mind experiments were undertaken to determine the effects of temperature upon embryonic development of insects.

TABLE I.

SHOWING SUMMARY OF DATA ON EFFECTS OF TEMPERATURE ON O_2 CONSUMPTION DURING EMBRYONIC DEVELOPMENTS OF *S. cecropia.*

Temp.	Manner of Calculation.	Total No. of		Milligrams of O_2 Used per gram of Live Weight per Hour by Days.								
		Masses.	Eggs.	1	2	3	4	5	6	7	8	9
28	Exclusive of shell	11	534	.345	.359	.500	.615	.632	.684	.860	1.262	1.817
28	Inclusive of shell	11	534	.260	.320	.381	.480	.500	.520	.672	.985	1.420
34	Exclusive of shell	4	213	.347	.415	.550	.653	1.150	1.660	2.560	3.460	
34	Inclusive of shell	4	213	.287	.321	.420	.525	.900	1.302	2.000	2.710	

TABLE II.

SHOWS THE MILLIGRAMS OF O_2 CONSUMED PER GRAM OF LIVE WEIGHT (EXCLUSIVE OF SHELL) PER HOUR.

Name of Insect.	Total No. of										
	Egg Masses.	Eggs.	1	2	3	4	5	6	7	8	9
A. tristis.	8	606	.383	.580	.890	.985	1.262	2.000	2.800		
S. cecropia.	11	534	.345	.350	.500	.615	.632	.684	.860	1.262	1.817
T. luna.	9	501	.397	.410	.421	.650	1.270	2.000	2.800		
P. ainsliei.	4	630	1.400	1.500	1.800	2.200	3.000	6.000			

The same eggs were used to make the determinations at both temperatures. After sufficient acclimatization, two to five hours, depending on the size of the animal chamber, the manometer was closed and at the end of two hours the reading was made. In like manner the reading for the next temperature was made and the eggs returned to the incubator at 28 degrees until the next day.

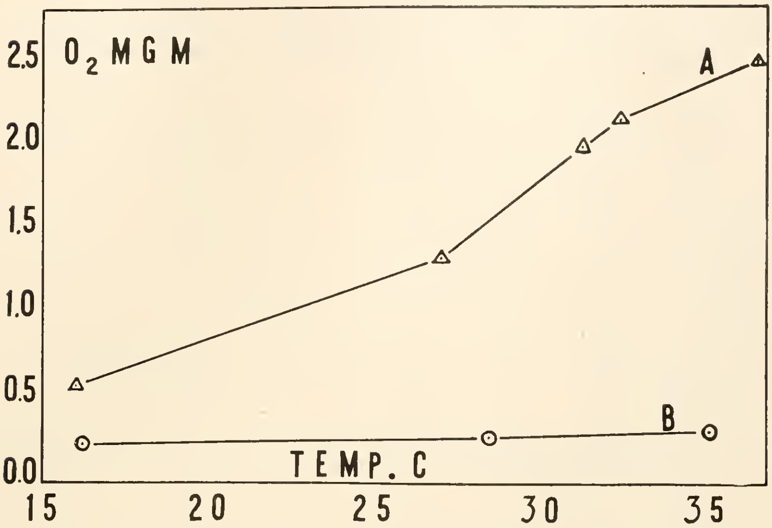

FIG. 1. Showing the effects of temperature on the O_2 consumption of *T. luna* eggs. (*A*) last day of incubation; (*B*) first day of incubation.

The results of these experiments are shown graphically in Fig. 1.

It is observed from Fig. 1 that the effects of temperature on O_2 consumption are very slight during the first day of incubation and very pronounced during the last day. In order to determine the effect of temperature on O_2 consumption during the entire incubation period daily determinations were made on two series of *S. cecropia* eggs at 28 and 34 degrees C. respectively. These results are summarized in Table I. and shown graphically in Fig. 2.

For comparative purposes the eggs of four species of insects were run at 28 degrees C. and the rate of O_2 consumption thus determined is shown graphically in Fig. 3 and summarized in Table II.

FORMATIVE PERIOD.

Upon examination of Figs. 2 and 3 we note that during the early part of the incubation period temperature has very little stimulation on the O_2 consumption but as the incubation period progresses its effects become pronounced. This is in accord with and substantiates the existing theory which states *that during early embryonic development there is a formative period during which metabolic activity is comparatively low and only influenced slightly by environmental changes.* Mention has been made, above, of the explanation offered by Fink for the variation in the length of this formative period among different species of insects. In the case of *S. cecropia* and *T. luna,* both species laying eggs on foliage, the formative period is somewhat lengthened. This is contrary to Fink's explanation. Data presented in this paper shows the length of the incubation period to be a greater

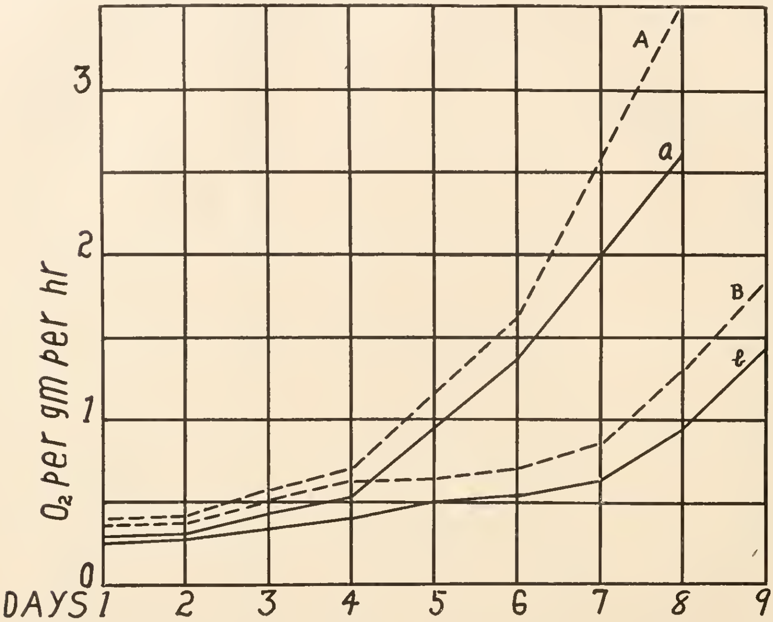

FIG. 2. Shows the effects of temperature on O_2 consumption during the entire incubation period of *S. cecropia.* (*A*) exclusive of shell 34° C., (*a*) same as above but including shell; (*B*) exclusive of shell 28° C., (*b*) same as above, but including shell.

factor in determining the length of the formative period than the type of place where the eggs chance to be deposited. Fig. 3 bears out this explanation. A study of Fink's curves will show that they too substantiate the explanation here offered.

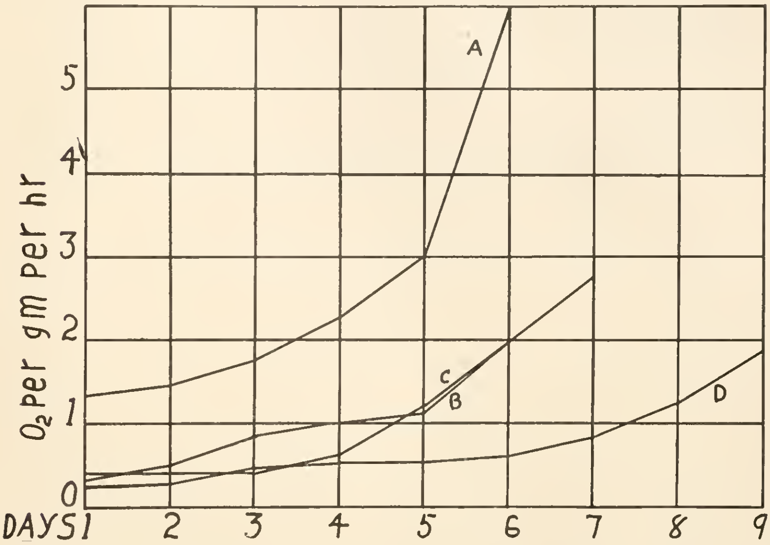

FIG. 3. O_2 consumption of eggs of (*A*) *P. ainsliei*, (*B*) *A. tristis*, (*C*) *T. luna*, and (*D*) *S. cecropia*.

SUMMARY.

From a preliminary study of the factors accompanying and influencing metabolism as determined by the O_2 consumption during embryonic development made on four species of insects the following conclusions are drawn:

I. The weight of the egg shell is an important factor and should be taken into consideration.

II. The effects of temperature are not as pronounced during the formative period as during the period of late incubation.

III. The explanation offered for the variation in the length of the formative period is the length of the incubation period and not the place where the eggs chance to be laid as has been suggested.

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BIOLOGICAL BULLETIN

THE INFLUENCE OF MOLDS ON THE GROWTH OF LUMINOUS BACTERIA IN RELATION TO THE HYDROGEN ION CONCENTRATION, TOGETHER WITH THE DEVELOPMENT OF A SATISFACTORY CULTURE METHOD.

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At the Marine Biological Laboratory, Woods Hole, during August and September, 1927, it was observed that cultures of luminous bacteria (*Bacillus Fischeri, Beijerinck, Migula*) tended to deteriorate rapidly, the deterioration being progressive, so that finally subcultures were made daily, the luminescence becoming steadily less, and the culture was finally lost. A fresh culture was obtained by plating out luminous material of the same stock, recovered from an old Petri dish culture contaminated with mold. This culture grew vigorously for a time, and then degenerated. Since the bacteria in contact with the mold continued to grow and glow for some time, new cultures were isolated when necessary.

These bacteria were being used for physiological experimental material, and it was considered necessary to learn the reason for the deterioration of the cultures and devise a cultural method by which bacteria of the same strain could be maintained in vigorous condition throughout any given series of experiments. Luminous bacteria live normally in sea water, which is maintained constantly in a fairly definite alkaline pH range. They are considered to grow best on culture media of about the same pH value as the sea water. All of these cultures were grown on the same medium, supposedly of the proper pH, and it was suggested by Professor Harvey that the trouble might be due to insufficient alkali reserve, the acid produced by the bacteria rapidly lowering the pH of the medium to a value unfavorable to their growth. The influence of the mold in causing continued light and growth might be due to alkali production. This explanation was favored by the result of pouring a solution of $M/2$ NaCl to which Clark's phosphate buffer, pH 8.0, had been added, over the surface of several Petri dish cultures which had ceased to glow. One, in which the light had been out only a few hours, again began to glow, and the luminesceice lasted for over eight hours. Others, in which the light had been extinct for longer periods, were not revived.

Friedberger and Doepner (1907) had studied the influence of various molds on the light intensity of cultures of luminous bacteria. They grew molds in bouillon, filtered the bouillon, and used this material in making up culture media. They found a greater intensity of light in cultures grown on these media than on controls prepared with ordinary bouillon. The one difference which they could establish between ordinary bouillon and bouillon in which mold had been grown was an increased alkalinity in the latter. Their figures show that 10 cc. normal bouillon neutralized .4 cc. $n/10$ NaOH to phenolphthalein, while 10 cc. of their "mold bouillon" neutralized .2 to .4 cc. $n/10$ H_2SO_4 to phenolphthalein. They arrived at the conclusion that the greater intensity of the light of cultures grown on "mold bouillon" was due in part to the increased alkalinity, and in part to "other properties" of the mold.

Molisch (1912) had shown that in general the intensity of light of cultures of luminous bacteria depended on the rate of growth. It is the opinion of the writer, for reasons given below, that the only cause for the increase in intensity of light and length of life of cultures of luminous bacteria grown in contact with mold is that of alkali production by the mold, which thus acts as an alkali reserve.

A series of experiments using solutions of $M/2$ NaCl plus Clark's phthalate, phosphate, and borate buffers, found to be non-toxic, showed that these bacteria glowed brightly in the pH range 5.7 to 8.7, the luminescence lasting for over an hour.

(Observations were not made after more than an hour had elapsed.) Below pH 5.2 the light lasted only a few seconds, above pH 9.0 for three minutes or less. The pH range in which growth can be expected lies then between 5.7 and 8.7, pH values outside this range being productive of rapid injury.

The culture medium in use was a peptone, beef-extract, glycerine agar, made up in sea water, the pH being adjusted to 8.2 with NaOH. As these bacteria live normally in an environment containing NaCl in about one half molecular concentration, favorable conditions are provided for the use of buffer mixtures. Molisch (1912) had shown that a number of salts other than NaCl might be used in culture media for luminus bacteria. A culture medium was made up in which one fifth mol of secondary potassium phosphate in 500 cc. distilled water was substituted for one half of the sea water. After sterilization the pH was adjusted with NaOH to 8.2. Separate lots of the same batch were colored with the Clark and Lubs selection of indicator dyes, covering the pH range from 1.2 to 9.8. Cultures were started on slants prepared from these media, six tubes of each being inoculated with luminous bacteria and three of each six being inoculated also with a common mold at one end of the slant. (The mold used was kindly identified for me by Dr. Charles Thom, as *Penicillium* sp., in the same section with *P. commune* (Thom) and *P. solitum* (Westling).) These were all allowed to develop somewhat below room temperature for two weeks.

Some of the indicators used were accumulated by the bacteria. These are being studied further to determine whether they penetrated the cell, or were merely adsorbed on the surface. They were of little value for this study, since not enough dye was left in the medium to indicate its pH value. However, in the case of several of these, the pH was indicated roughly by the color of the dead bacteria, which was not markedly different from the medium. With brom cresol green (yellow at 3.8, blue at 5.8), the dead bacteria near the mold were a more intense blue than elsewhere, and the acid range of the indicator had not been reached anywhere in the slant.

On the chlor-phenol red slants (yellow at 5.2, red at 6.8) the color of the medium indicated that the pH had been reduced to

$5.4 \mp .2$. The pH of the medium near the mold was well above the alkaline range of the indicator.

On the cresol red slants (yellow at 7.2, red at 8.8) the color of the medium indicated pH below the range of the indicator except near the mold, where a pH of $8.6 \mp .2$ was indicated. The results with meta cresol purple were about the same. With thymol blue, the color of the indicator was masked by the color of the medium at the critical value, and it was of no value.

On the same date six cultures were started on medium of the same batch without addition of indicator. At the end of two weeks all were alive and glowing brightly. These cultures decreased slowly in brilliance during the next month, but were still glowing faintly at the end of six weeks, and viable transfers were made at the end of the seventh week. The final death of these cultures appeared to be caused by the drying up of the medium.

As a further check on the alkali influence, several cc. of *M*/NaOH was introduced at the bottom of each of six slants of unbuffered medium colored with brom thymol blue, and an equal number without indicator. Streaks made on these slants developed rapidly on the upper half of the slant, away from the alkali, and grew well, the cultures on the uncolored medium lasting for several weeks (average of six, 22.2 days), until the alkali was exhausted. On one of these, more alkali was added and a fresh inoculation made, the growth lasting this time for less than a week. It was observed that no growth took place below the line which marked the limit of diffusion of strongly alkaline NaOH. This limit was well marked on the uncolored medium by the precipitation of magnesium hydroxide.

The most characteristic activity of luminous bacteria seems to be that of acid production. They are killed in a few days in their own acid if some method of neutralization or removal is not employed. In their natural environment the excess acid would simply diffuse into the surrounding sea water, but within the limits of the test tube this cannot occur. The base used in the culture medium was NaOH, which in contact with carbon dioxide becomes $NaHCO_3$, and since $NaHCO_3$ in the concentration used (.01 *M*) is not particularly acid when saturated with carbon dioxide, it is not likely that the acid limiting their growth is carbon dioxide. That it is a non-volatile acid is shown by the following experiment:

A constant stream of sterile air was drawn in series through three bottles of slightly buffered culture medium colored with cresol red. The first of these was the control, without bacteria. The other two were inoculated with luminous bacteria. At the end of 24 hours the control was red, as at the start, and uncontaminated as shown by the absence of turbidity, and this condition lasted until the close of the experiment. The two inoculated bottles at the end of 24 hours were down to about pH 7.4. Enough NaOH was added to the third bottle to restore the original pH of approximately 8.2, and this was repeated every two hours until the close of the experiment. At the end of 36 hours, the PH in bottle No. 2 was down to about 5.5 (determined by withdrawing some of the material and testing with other indicators) and the light was extinct. In bottle No. 3, in which pH 8.2 was maintained, the bacteria continued to glow for another 24 hours, when the light failed, due presumably to failure of the food supply. Carbon dioxide and any other acids volatile at room temperature (if any were formed) would have been swept out by the stream of air, leaving behind the non-volatile acid. This is probably lactic acid.

Other culture media were tried in which calcium and barium carbonates were employed as buffers, and also higher concentrations of K_2HPO_4 and sea water, and lower concentrations. Luminous bacteria can tolerate a considerable range of salt concentration. It was found that on phosphate buffered media where the total salt concentration was greater than in sea water, but not in excess of molar concentration, growth was slower than on media of the proper concentration, and the tendency to diffuse growth was absent. The resultant crowding gave the streaks a fictitious brilliance for a few days, after which the light intensity decreased to a low value. These cultures were viable for fairly long periods of time, average 21 days, but not as long as cultures on media of the proper salt concentration. When media of lower total salt concentration (as about ¼ molar) were used, there was an initial rapid growth, accompanied by flowing over the surface of the medium, and a rapid decay, so that such cultures were viable for only a few days, the average of six cultures being five days. Since a heavy precipitate of calcium and magnesium phosphates was formed when the phosphate buffer was added to sea water, media were prepared containing various concentrations of NaCl, from .25 *M* to .75 *M*, as substitutes for sea water, but these were unsatisfactory, the best of them lasting for only 14 days.

On medium buffered with calcium carbonate, growth was vigorous, but the life of the cultures was less than with the best of the phosphate buffer mixtures. The average length of life of eleven cultures without indicator was 17.8 days. Curiously enough, the death of these cultures was due to excess alkalinity. The initial growth was rapid, but on the third or fourth day there was a decrease in brilliance of light and a slowing down of growth, caused by the rapid diffusion of the acid through the agar, using up the small amount of calcium hydroxide in solution. This was followed by an increased brilliance and renewed growth as the pH rose again, due to the solution of more calcium hydroxide (produced by hydrolysis from the calcium carbonate), and its diffusion through the medium. The calcium salt of the acid produced by the bacteria is much more soluble than calcium carbonate, and is evidently hydrolyzed in solution, for the medium becomes steadily more alkaline until the alkaline range of the available indicators is passed. Since the bacteria are soon killed by alkali above pH 9.0, the limiting value is passed, and luminescence ceases. This can happen only when the calcium carbonate is in excess. When the pH of the medium was adjusted with calcium carbonate, and the excess carbonate filtered off, initial growth was rapid, but the decline following it continued until the death of the culture occurred on the sixth day (average of six cultures), caused by acidity as shown by the use of a suitable indicator.

On the medium prepared with barium carbonate from which the excess carbonate was filtered off, the initial fair growth was followed by a rapid decline, the average length of life of 14 such cultures being 6.5 days. When an excess of barium carbonate was present, the initial growth was fair, and slowly decreased, the cultures growing steadily more alkaline, the average length of life of 14 cultures being 17.5 days. Although theoretically about the same pH value should be produced by barium and calcium carbonates, in practice the medium prepared with barium carbonate was always the more alkaline, and was too alkaline for good growth of the bacteria. The vigorous growth obtained on calcium carbonate was never obtained on media prepared with barium carbonate.

The medium prepared with calcium carbonate has the advantage that no adjustment of pH is required, the hydrolysis of the carbonate giving approximately the right value. It is by far the best buffer substance to use, both for slants and for Petri dish cultures. The medium should contain 20 grams "Bacto" nutrient agar, 10 cc. glycerine, and 5 grams calcium carbonate per liter, made up in sea water. If a transparent medium is desired, the phosphate buffer mixture with the same amount of nutrient substance, made up in sea water and filtered, may be used. The optimum pH value for this medium, probably about 8.6, may be secured by titrating the hot medium by the drop method until a good red is secured with cresol red, and a barely perceptible color with thymol blue. When one fifth mol of buffer is added to sea water, the average life of cultures emitting strong light is 18 days. After this time, very little light is emitted, but viable transfers may be made for several weeks.

Of the indicators used, several appeared to be slightly toxic to the bacteria, but the evidence on this point is inconclusive.

SUMMARY.

The influence of molds on the length of life of cultures of luminous bacteria may be simulated by the use of buffer mixtures, or by supplying fresh alkali continually. The maximum alkalinity produced in these experiments by the influence of *Penicillium* sp. was pH $8.6 \mp .2$. Degeneration of cultures of luminous bacteria may be caused by growth on media insufficiently alkaline, or so slightly buffered that it soon becomes acid. Diffuse growth and spreading over the surface of the slant is caused by too low salt concentration. Long life of cultures may be secured by growing on media sufficiently alkaline, and sufficiently buffered to resist rapid change by the acid production of the bacteria, which are killed by their own acid at about pH 5.6.

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THE SEX RATIO IN *PEROMYSCUS.*

JOHN J. KAROL.

The data herein presented are based on the records of breeding experiments with *Peromyscus*, conducted by Dr. F. B. Sumner. In an earlier paper [1] the sex ratio in *Peromyscus* was discussed at considerable length and data covering the years 1915–1921 inclusive were presented. The present report is based on the records of births from 1922–1926 inclusive. The material is made up partly of the various mutant strains of the *maniculatus* series, variously hybridized and partly of the three subspecies of *Peromyscus polionotus*, *P. p. polionotus*, *P. p. leucocephalus,* and *P. p. albifrons,* both pure and hybrid. No attempt will be made to give comprehensive interpretation of the findings but reference may be made to the paper cited above for more detailed discussion.

I take this opportunity of acknowledging my indebtedness and sincere thanks to Dr. F. B. Sumner who suggested the subject and under whose general guidance the work was carried out.

The influences which might affect the sex ratio in *Peromyscus* were considered in this treatment of the data to be (1) season, (2) size of litter, (3) race, (4) hybridization.

The total number of broods recorded in the records from 1922 to 1926 is 760, comprising 2,522 young, or an average of 3.32 mice per brood. According to sex these were distributed as follows:

Males	1,316
Females	1,114
Sex undetermined (dead or escaped)	61

The sex ratio (number of males per hundred females) for those of known sex is 114.93 ± 3.19.[2] It is interesting to note here that the sex ratio for the data from 1915 to 1922 was 97.37 ± 1.93.

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[1] Sumner, McDaniel and Huestis, BIOL. BULL., No. 2, 1922.

[2] The probable error here employed is $\pm 67.45\ (1 + R)\sqrt{\frac{R}{n}}$, in which $R =$ sex ratio.

Since the number of individuals considered here is about half as great as that in the previous paper on *Peromyscus*, we shall present the data of this later period only for what they may be worth. At the suggestion of Dr. Sumner it was considered permissable to combine these additional data with the earlier records and thus, in a sense, bring some of the results on the sex ratio of *Peromyscus* up to date.

The total number of broods in the combined data from 1915 to 1926 is 2,321, comprising 7,547 young, or an average of 3.25 mice per brood. According to sex these were distributed as follows:

Males	3,597
Females	3,492
Sex undetermined (dead or escaped)	458

The sex ratio for the combined data is thus 103.01 ± 1.64.

SEASON.

The following table gives the sex ratio for each month of the year and also the number of individuals upon which this ratio is based. The table contains the total data for the years 1922–1926.

January (162)	123.61 ± 13.29
February (154)	120.59 ± 13.21
March (360)	106.43 ± 7.64
April (290)	114.39 ± 8.94
May (390)	110.50 ± 7.65
June (277)	123.77 ± 10.12
July (184)	111.90 ± 11.30
August (220)	143.68 ± 13.50
September (140)	140.35 ± 19.42
October (143)	92.96 ± 10.67
November (109)	76.67 ± 10.15
December (68)	120.00 ± 19.30

As it is obvious from the graph that the differences between the consecutive months are of little significance we may combine our monthly birth records into four seasons of three months each. In both the earlier data alone and in the combined data we may distinguish two high periods and two low periods annually. The sex ratios for these four periods applied to the later data are as follows:

(1) February–April	111.86 ± 5.43
(2) May–July	114.99 ± 5.36
(3) August–October	126.55 ± 7.77
(4) November–January	105.56 ± 7.92

The greatest difference between two of these ratios is that between the third and fourth periods This difference is 20.99 ± 11.09.

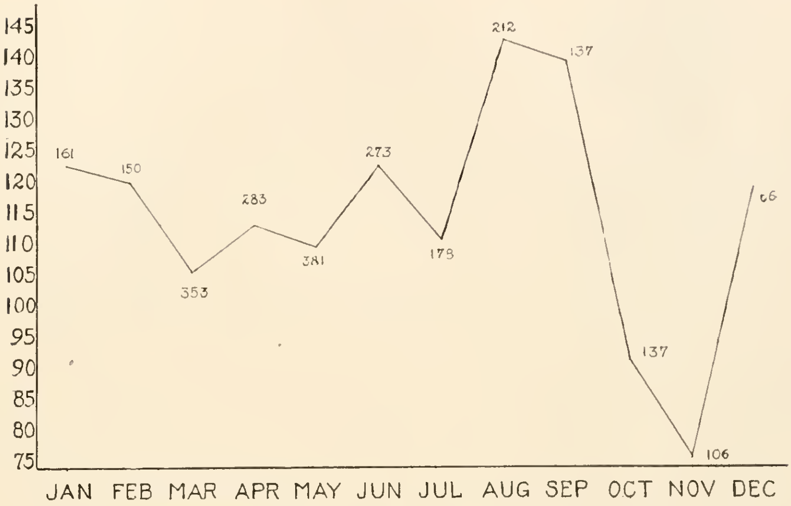

FIG. 1. The sex ratio of *Peromyscus* for each month of the year computed for the data from 1922 to 1926. The figures along the graphs denote the number of individuals born during each month of the year.

Grouping the same data according to the seasons of the year, as employed by King and some others, we get the following sex ratios:

Spring (March–May)	110.12 ± 4.90
Summer (June–August)	126.28 ± 6.71
Autumn (September–November)	102.13 ± 3.08
Winter (December–February)	121.76 ± 8.53

Here the greatest difference is between summer and autumn, being in this case 24.15 ± 7.38. Inasmuch as our figures are small we make no attempt to attach any particular significance to these values but we may say in passing that they are of the same order of magnitude as the findings of King[1] in the Norway rat. In both we find a maximum in summer followed by a minimum in autumn.

Combining the earlier data (1915–1921) with these additional data we get the following monthly sex ratios:

January (395)	103.53 ± 7.41
February (469)	99.54 ± 6.17
March (1,129)	106.26 ± 4.30
April (660)	113.65 ± 6.20
May (967)	101.13 ± 4.61
June (707)	100.59 ± 5.29
July (592)	98.11 ± 5.74
August (818)	113.46 ± 5.46
September (617)	108.45 ± 6.03
October (564)	103.37 ± 6.02
November (367)	78.12 ± 5.76
December (279)	96.38 ± 7.93

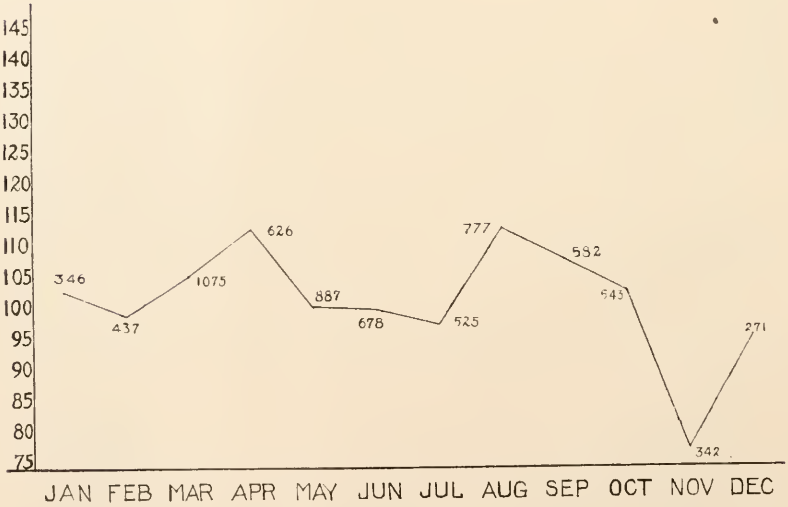

FIG. 2. The sex ratio of *Peromyscus* for each month of the year computed for the combined material from 1915 to 1926. Numbers along graphs indicate numbers born in each month.

Here, as in the earlier data alone, we find two annual maxima, one occurring in March and April; the other from August to October. In the graph we have the appearance of a fairly well marked biennial rhythm.

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[1] *Archiv für Entwickungsmeckanik,* 1927, 61.

Now grouping the combined data according to 3-month periods we find the following sex ratios:

(1) February–April	106.96 ± 3.07
(2) May–July	100.19 ± 2.96
(3) August–October	107.87 ± 3.36
(4) November–January	91.80 ± 4.01

The difference between the third and fourth periods is 16.07 ± 5.23 and may be considered of probable significance according to the conventional statistical standard. These figures still show a rather marked biennial rhythm despite the fact that the later data showed reversed relations for the February–April period.

Again, if we regroup the combined data by the ordinarily recognized seasons the figures become:

Spring (March–May)	106.22 ± 2.78
Summer (June–August)	104.76 ± 3.18
Autumn (September–November)	97.43 ± 3.45
Winter (December–February)	100.00 ± 4.18

Here the greatest difference, between spring and autumn, is 8.79 ± 4.43 and of no probable significance. Likewise the biennial rhythm, apparent in the case of the later data seems to have been eliminated by the addition of the earlier data. This, we may say, is typical of the conflicting results pervading the entire literature on the sex ratio.

In the previous paper on the sex ratio in *Peromyscus* it was stated that the records were "unfortunately not adapted to revealing definite periods of increased or diminished reproductive activity, since the matings were to a large extent controlled in accordance with the demands of the breeding experiments." Since this statement is equally applicable to the later data, we wish to stress the point that only the number of matings was controlled and we cannot understand how this could possibly affect the normal seasonal trend of the sex ratio[1]

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[1] *Cf.* King, 1927.

SIZE OF THE BROODS.

The mean size of the 760 broods considered in the later data is 3.32. The following table gives the sex ratios for mice belonging to broods containing from one to seven individuals respectively. Double broods or broods in which individuals of unknown sex are known to have died have been excluded.

No. in Brood.	Males.	Females.	Ratio.
1	17	12	141.67 ± 36.07
2	119	103	115.53 ± 10.44
3	423	336	125.89 ± 6.25
4	352	316	111.39 ± 5.83
5	141	129	109.30 ± 9.02
6	59	31	190.32 ± 28.36
7	18	17	105.88 ± 24.17

Summarizing the combined data we get the following table for the sex ratios according to the size of the brood:

No. in Brood.	Males.	Females.	Ratio.
1	81	73	110.96 ± 12.09
2	351	355	98.87 ± 4.96
3	1,047	993	105.44 ± 3.18
4	1,029	983	104.68 ± 3.18
5	405	385	105.19 ± 4.97
6	159	111	143.24 ± 11.96

Considering either the single or combined data we can find no significant differences in the sex ratios of various sized litters and we can only conclude that the size of the brood does not seem to have any well-defined relation with the sex ratio in *Peromyscus*.

Separate calculations were made for the litters in which no deaths were recorded and for the litters in which deaths are known to have occurred. In the later data we find the sex ratio for incomplete broods, comprising 43 broods, to be 83.64 ± 11.26. For the 673 complete broods the sex ratio is 118.09 ± 3.38—the difference between incomplete and complete broods being 34.45 ± 11.76. While this difference is large enough to be of interest we cannot attach any great significance to it inasmuch as only 43 incomplete broods were considered. In the combined data we find sex ratios of 91.45 ± 4.76 and 104.65 ± 1.79 based on 309 and 1,974 broods for the incomplete and complete broods respectively. Thus we do find a difference between the sex ratios of complete and incomplete broods but we do not feel justified in regarding it as significant in view of the meagre record of identified dead.

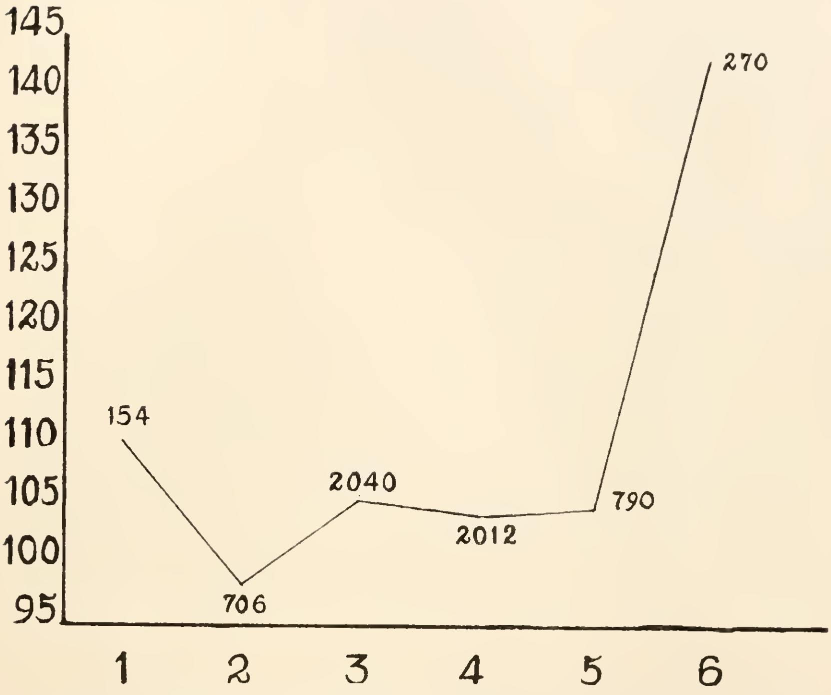

FIG. 3. Variations in the mean sex ratio, according to the size of the broods. Numbers along graphs indicate numbers of individuals.

Combinations of the Sexes in Individual Broods.

It is interesting to consider the possible tendency of members of a litter to agree with one another in respect to sex, that is, whether or not we encounter broods consisting entirely of the same sex more frequently than would result from chance. In the following table, using the combined data, we have arranged broods of each size in groups according to the number of each sex present. For example, broods of three present four possible combinations: 3 ♂, 2 ♂ + 1 ♀, 1 ♂ + 2 ♀, 3 ♀. The actual number of complete broods containing a given combination of males

TABLE I.

NUMBER OF BROODS CONTAINING EACH POSSIBLE COMBINATION OF MALES AND FEMALES.

Number in Brood.														
1	Combinations.......						1♂		1♀					
	No. of broods (actual)						81		73					
	Expected number....						78		76					
2	Combinations.......					2♂		1♂, 1♀		2♀				
	No. of broods (actual)					83		185		85				
	Expected number....					91		176		86				
3	Combinations.......				3♂		2♂, 1♀		1♂, 2♀		3♀			
	No. of broods (actual)				92		260		251		77			
	Expected number....				89		259		251		81			
4	Combinations.......			4♂		3♂, 1♀		2♂, 2♀		1♂, 3♀		4♀		
	No. of broods (actual)			30		140		183		123		27		
	Expected number....			33		130		188		122		30		
5	Combinations.......		5♂		4♂, 1♀		3♂, 2♀		2♂, 3♀		1♂, 4♀		5♀	
	No. of broods (actual)		8		28		45		48		22		7	
	Expected number....		5		26		50		49		24		5	
6	Combinations.......	6♂		5♂, 1♀		4♂, 2♀		3♂, 3♀		2♂, 4♀		1♂, 5♀		6♀
	No. of broods (actual)	0		5		16		13		8		3		0
	Expected number....	1		4		11		14		10		4		1

and females and the "expected" number to the nearest integer are computed. Since the percentage of males in the combined data is 50.74, I have computed these last figures by expanding the binomial $(1.015 + .985)^n$. In the case of an equality ratio we should use the ordinary formula for probability, *e.g.*, $(1 + 1)^n$. Considering the comparatively small number of broods present in most of the groups we find a rather close agreement between the actual and the expected figures for all of the broods in which all members were of the same sex. The actual number of such homosexual litters, among broods containing from 2 to 6 individuals inclusive, was 409; while the most probable number on the assumption of purely random sex-production, was 422. If we consider fractions (a more exact procedure) this last figure becomes 420. In the earlier data alone a closer agreement than this was found, the figures being 276 and 274 for the actual and expected number of broods respectively. It would appear that the distribution of the sexes in single broods follows the laws of chance and there seems to be no tendency for fetuses (or germ cells) developing in the same parents at the same time to give rise to organisms of the same sex. We may likewise reiterate the conclusion of Sumner, McDaniel and Huestis, namely; "the non-occurrence of polyembrony or true twinning, at least with sufficient frequency to affect the results."

RACE.

For the later data we have computed the sex ratio separately for the "pure" (non-hybrid) polionotus series and in the following table we have listed in addition the sex ratios for some of the other geographic races (subspecies) as computed by Sumner, McDaniel and Huestis.

Subspecies.	Males.	Females.	Ratio.
polionotus	120	89	134.83 ± 13.15
gambeli (La Jolla)	770	840	91.67 ± 3.07
sonoriensis	350	373	93.83 ± 4.70
rubidus	150	124	120.97 ± 9.91

The difference between "*polionotus*" and "*gambeli*" is 43.16 ± 13.50 and may possibly be regarded as significant. But we cannot say definitely that these figures imply the existence of any actual racial differences with regard to the sex ratio.

It is interesting to also observe here that in the subspecific hybrids of *Peromyscus polionotus* we find a sex ratio of 114.61 ± 5.79 while the subspecific hybrids considered in the earlier report (mainly *P. maniculatus*) give a mean sex ratio of only 104.76 ± 3.41.

Parkes[1] briefly summarizes the data of many workers on specific variations in the sex ratio in man and other mammals.

Hybridization.

In 235 broods comprising 735 individuals of F_1 hybrids in the later series we find a sex ratio of 114.61 ± 5.79. While this is lower than the ratio for the pure "*polionotus*" stock (see p. 159), we cannot attach any significance to the latter figures since they are so small. For the same reason we do not feel justified in combining the later group with the earlier, in a comparison of pure and hybrid ratios. We may say, however, that in the earlier series alone the difference between the ratios for pure and hybrid stock was found to be 11.49 ± 4.1, the hybrid series giving the higher ratio. These results are in agreement with the conclusions reached by other workers, *e.g.*, Pearl (1908), King (1911), and Little (1919), that hybridization "per se" may result in raising the sex ratio.

The Year.

The sex ratios and the number of individuals upon which they are based for the year 1922–1926 are as follows:

1922	(296)	106.34 ± 8.33
1923	(355)	106.43 ± 7.64
1924	(519)	120.80 ± 7.27
1925	(966)	113.93 ± 5.03
1926	(386)	125.60 ± 8.80

Although it is quite evident that there are no significant differences here it was thought worth while to present the figures in view of the fact that the earlier data on *Peromyscus* (1915–1921) showed such marked yearly variations. While these results were inexplicable, they were statistically speaking, the most significant of all and the likelihood of obtaining one of the differences by "accident" was less than one in 40,000. It was further proven that these differences were "not due either to the seasonal distribution of births, to the preponderance of hybrid births in one year as compared with another, or to the operation of any of the other factors previously considered."

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[1] A. S. Parkes, "The Mammalian Sex Ratio," *Biol. Review,* Vol. II., No. 1, Nov., 1926.

Inasmuch as it is evidently exceedingly difficult to correlate the annual variation in the sex ratio with any known influences, accurate data on the subject are generally lacking. Of course it is not impossible that the most "significant" figures may result from chance.

SUMMARY.

Data have been presented based upon 2,522 deer mice as recorded during the breeding experiments of Dr. Sumner, from 1922 to 1926. Earlier records (1915–1921) were added to the above and the combined data have also been presented.

The following results seem to be of most importance.

1. The mean size of 760 broods in the later records is 3.32. For the combined data comprising 2,321 broods the mean size is 3.25 mice per brood.

2. The sex ratio for the later data is 114.93 ± 3.19; while that for the entire lot is 103.01 ± 1.64.

3. Considering the possibility of a seasonal cycle in the proportion of males and females born, we can only say that we find in the later data a maximum sex ratio in the August-September period followed by a minimum during October and November. In the combined data we find two annual maxima, one occurring in March and April, the other from August to October, and hence a fairly well marked biennial rhythm. Grouping the combined material according to 3-month periods we find in one arrangement that the biennial rhythm is practically eliminated while in another it is rather well marked. The existence of a seasonal cycle in the sex ratio of *Peromyscus* is not definitely proved.

4. The size of the brood in the combined material does not seem to have any well defined relation with the sex ratio in *Peromyscus*.

Although we find a difference between the sex ratio of complete and incomplete broods we cannot regard it as significant in view of the meagre records of identified dead.

5. When the number of each possible combination of males and females, in broods of each size, is compared with the number expected according to chance, the conformity is found to be, on the whole, very close. For example, if we compare the actual and expected totals for all of the broods in which all members were of the same sex we find 409 as the actual number and 420, the "expected" number. Thus there is no preponderant tendency toward the production of homosexual litters and thus the non-occurrence of polyembrony or true twinning to any great extent.

6. While the sex ratio for the three subspecies of *polionotus* is "significantly" higher than that for other pure races of *Peromyscus* we cannot say definitely that these figures imply the existence of any actual racial differences with regard to the sex ratio in *Peromyscus*. The sex ratio of *polionotus* hybrids is likewise considerably higher than that of other *Peromyscus* hybrids which have been studied.

7. No significant yearly variations were found in the sex ratio of *Peromyscus* from 1922 to 1926.

COLD HARDINESS IN THE JAPANESE BEETLE, *POPILLIA JAPONICA* NEWMAN.

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Cold hardiness, or the ability of an organism to withstand low temperature may be considered from two points of view, (1) cold hardiness to the intensity factor or the ability to survive extreme low temperatures, and (2) cold hardiness to the quantity factor or ability to withstand long periods of low temperature. By low temperature is meant, temperature below that required for normal development.

The Japanese beetle, which was introduced into the United States about 1916, can be secured in large numbers, thus making intensive study possible. This insect represents a type of ecological group, the soil dwelling insects. It passes the winter in the larval stage; about 97 per cent. in the third instar; about 3 per cent. in the second. Cold hardiness to both the quantity and intensity factor of low temperature was studied. Both external and internal factors are involved in cold hardiness. These include such environmental factors as relative humidity and temperature, and such physiological conditions as nutritional state, health, blood conductivity and metabolic rate. Most of the work was done on larvæ. Some studies were made on adults and a few observations were made on cold hardiness in pupæ.

Methods and Apparatus.

Respiratory rate and quotient were determined by the modified Krogh manometer of Bodine and Orr (1925). Conductivity of blood and body fluids was determined by the ionometer, described by Gram and Cullen (1923). pH was determined with the type K potentiometer, using a small vessel capable of testing the pH of a drop. By this method several readings could be taken on the same larva. This method was described by Bodine and Fink

(1925). Occasionally a larva was found that would not bleed freely enough to give sufficient blood for a reading. Blood was usually taken from one of the feet. Relative humidity was maintained by pulling air over different concentrations of sulfuric acid by means of a suction pump.

Cold Hardiness to the Intensity Factor of Low Temperature.

In comparison with the oak borers previously studied by the author, Payne (1926), the Japanese beetles are less cold hardy and also exhibit less variation to low temperature. In Pennsylvania the most cold hardy Japanese beetle withstood — 28° C.; the most cold hardy oak borer — 47° C. The most cold hardy Japanese beetle collected in the field thus far withstood — 15° C.

Periodicity in cold hardiness to the intensity factor of cold is not as marked in the Japanese beetle as in the oak borers *Synchroa punctata* and *Dendroides canadensis*. Comparison of the three species in question tested at the same dates is shown in Table I.

Conditions other than seasonal which modify the cold hardiness of the Japanese beetle to the intensity factor of low temperature are (1) degree of dehydration, (2) disease incidence, (3) nutritional state, and (4) temperature at which the larvæ were kept. Although these larvæ are seldom collected in dry places normally, they are able to withstand a high degree of dehydration. Larvæ dried down to a pulpy condition in which the free water is reduced to a minimum are cold hardy to both intensity and quantity factors of low temperature. Severe dehydration is accompanied by a high death rate. Larvae can be dried down to one third of their body weight. In the dehydrated condition the Japanese beetle larvæ reach their greatest cold hardiness. Since eighty per cent. of dehydrated larvæ die the effect of dehydration may be considered highly selective, killing off those larvæ unable to hold water. Those larvæ capable of resisting dehydration are cold hardy. Relative humidity affects cold hardiness in a decided manner. The results of a series of different experiments with varying relative humidities is shown in Table II.

TABLE I.

Species.	September 29, 1926.			November 5, 1926.			December 11, 1926.		
	Mean Under-cooling.	σ.	C.V.	Mean Under-cooling.	σ.	C.V.	Mean Under-cooling.	σ.	C.V.
Synchroa punctata	−2.75	± .153	.056	− 7.5	±2.794	.374 −	−12.4	±3.422	.276
Dendroides canadensis	−8.45	±2.64	.313	−15.6	±4.087	.264 −	−17.34	±3.606	.208
Popillia japonica	−2.2	± .114	.0518	− 4.9	± .2156	.044	− 6.2	± .335	.054

Table II.

Conductivity of Blood of Japanese Beetle Larvæ Kept at Different Temperatures.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

0° C.	10° C.	20–22° C.	25° C.
.65	.6	.38	.33
.68	.61	.45	.35
.72	.604	.42	.38
.64	.58	.41	.39
.70	.604	.42	.40
.69	.55	.41	.41
.67	.58	.45	.375
.66	.6	.44	.39
.68	.6	.435	.40
.71		.46	.42
		.45	.39
		.43	.41
		.445	

Starvation at high temperatures, 20° C. or above, is fatal to the larvæ unless the relative humidity is kept high. When kept at high humidity, larvæ are able to withstand comparatively long periods of starvation. One hundred larvæ were kept without food for the month of May, 1927, but under conditions of 100 per cent. relative humidity or saturation. Each larva was placed in an individual vial and weighed before and after the starvation period. During the process they lost about one half of their body weight. None of them survived freezing, the lowest freezing point was — 1.7°C.; the highest — .65° C. Larvæ kept at + 10°C., or below their developmental temperature, lost one half of their body weight. Starvation conditions were assured by keeping the larvæ in sterile white sand kept moist with distilled water. About one fourth of these larvæ survived freezing. Changes in body weight under different conditions of starvation and dehydration are shown in Fig. 1. The effect of prolonged exposure to low temperature as well as starvation was involved in the experiment described above. The effect of different temperatures on cold hardiness as measured by blood conductivity is shown in Table III. Larvæ starved for one week at + 20° C. increased in cold hardiness. In general early stages of starvation are marked by an increase in cold hardiness, later

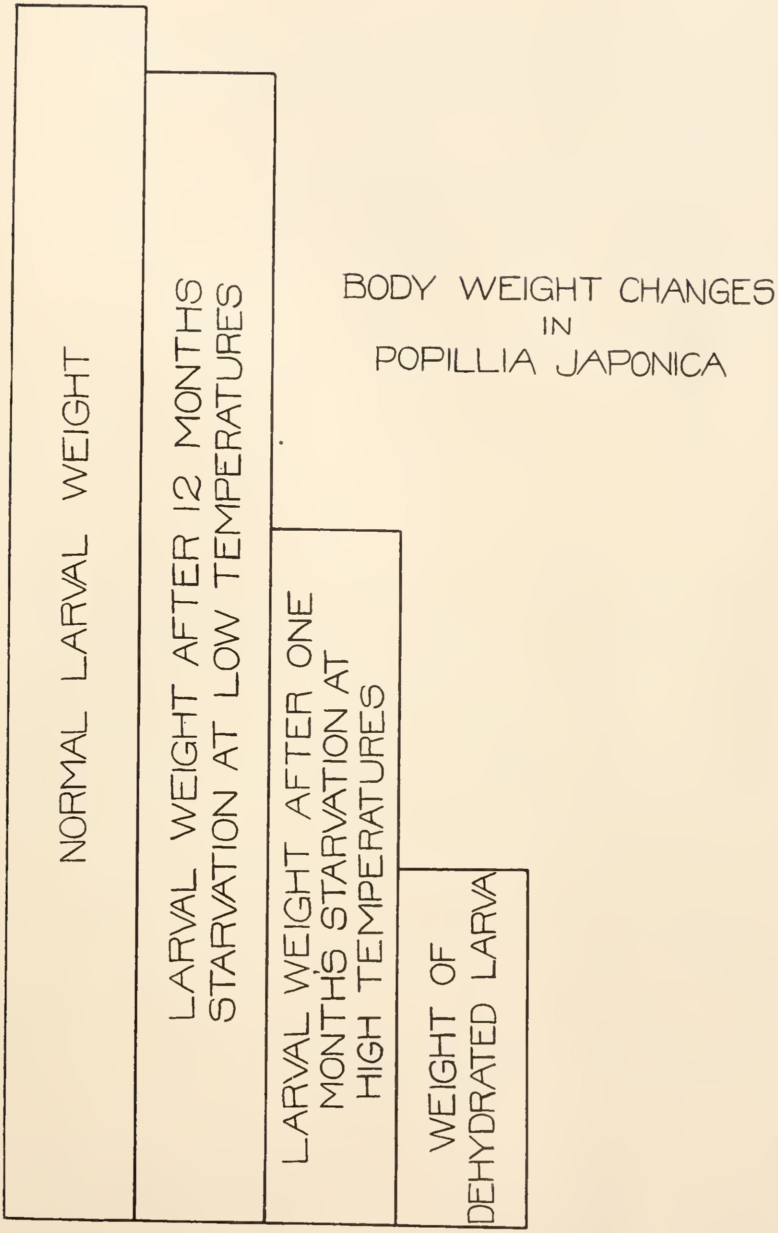
BODY WEIGHT CHANGES
IN
POPILLIA JAPONICA
NORMAL LARVAL WEIGHT
LARVAL WEIGHT AFTER 12 MONTHS STARVATION AT LOW TEMPERATURES
LARVAL WEIGHT AFTER ONE MONTH'S STARVATION AT HIGH TEMPERATURES
WEIGHT OF DEHYDRATED LARVA

FIG. 1.

TABLE III.

EFFECT OF DIFFERENT RELATIVE HUMIDITIES ON CONDUCTIVITY AT TEMPERATURE OF 22° C.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

Saturation.	80%.	50%.
.33	.46	.72
.38	.45	.75
.35	.47	.76
.39	.465	.78
.40	.44	.73
.41	.455	.77
.375	.46	.74
.39	.44	.76
.40	.455	.77
.375	.46	.76
.38	.45	.82
.32	.46	.77
	.445	.78
	.43	

stages by a decrease. The point of decrease in cold hardiness from starvation comes when the digestive tract clears. In connection with this observation it is interesting to note that freshly molted larvæ are unable to withstand freezing until they have eaten. Pre-pupæ with clear digestive tracts are not cold hardy.

The occurrence of wilt disease in many of the specimens collected in the field offered an opportunity for the study of the effect of this disease on cold hardiness. Larvæ were collected at the same date and subjected to the same conditions of temperature and relative humidity, only healthy larvæ were studied. No larva showing typical symptoms of wilt disease or polyhedralskränkheit was able to survive freezing. Since thermocouples used in diseased larvæ were difficult to sterilize and might infect healthy larvæ, cold hardiness was studied by measuring blood conductivity rather than freezing point depression. Conductivity decreases as the disease progresses. On the first day of apparent infection, conductivities of blood of diseased larvæ were below that of healthy larvæ. To produce such a marked change on the first day of infection, the causative organism must affect the blood very profoundly and very rapidly. On the other hand the change in conductivity may not be as rapid as it appears. The disease may be present in larvæ before it is detected by discol-

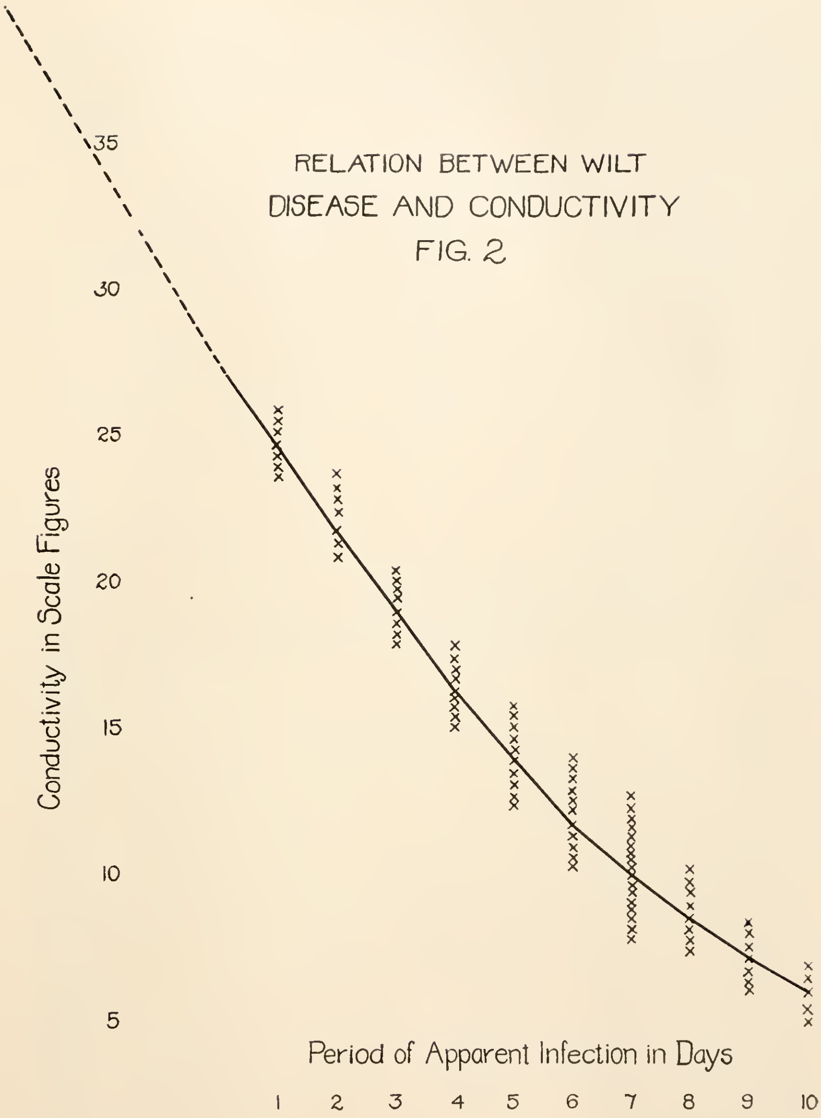

Fig. 2.

oration or wilting, and may be producing conductivity changes in the blood before other symptoms can be observed. A graph showing the relationship between day of apparent infection and blood conductivity is shown in Fig. 2. Table IV. shows the re-

TABLE IV.

CONDUCTIVITY OF HEALTHY AND DISEASED JAPANESE BEETLE LARVÆ.

(Conductivity shown in % NaCl equivalent uncorrected for protein.)

Wilt Disease.	Healthy.	Blackened by Freezing.
.21	.38	.6
.17	.4	.6
.10	.41	.604
.11	.4	.604
.04	.39	.58
.05	.375	.575
.07	.43	.61
.18	.425	.63
.12	.41	.62
.06	.39	.625
.13	.4	.64
.25		
.27		
.15		

sults of conductivity readings made on the blood of diseased larvæ in comparison with healthy ones.

Wilt disease is characterized by a pronounced blackening that precedes the final softening that occurs just before death. Blackening also has been observed when larvæ are frozen and thawed quickly. Blood from larvæ blackened after thawing always showed high conductivity. In these cases discoloration was believed to be due to changes in cell permeability releasing certain oxidative enzymes, which on escaping blackened the cells. The prothoracic segment is the first portion of the larvæ to discolor after freezing, both in the Japanese beetle and in the oak borers studied. Changes in permeability could be observed during the thawing process. Water apparently passes through the body wall where the chitin is thinnest. This water was frequently reabsorbed when the larvæ were kept under small bell jars. Larvæ losing water alone were generally able to survive freezing. When the fluid exhuding from the larva gave tests for amino-acids or proteins the larvæ always died. The exudate remained colorless for several days unless hydrogen peroxide was added, in which case it blackened quickly. Larvæ which showed the exudate after thawing were fixed and sectioned, but in these sections no gross differences from normal tissue could be detected. Broken cell walls were not in evidence. The direct cause of death from extreme low temperature has been interpreted as due to an irreversible change in permeability rather than to a breaking of the cell walls.

If larvæ capable of surviving low temperature are ground up and filtered and the filtrate precipitated with lead acetate, there occurs in the filtrate an enzyme capable of breaking proteins down to amino acids at low temperatures and of building up proteins from amino acids at high temperatures. A similar enzyme has also been found in tussock moth eggs. Reversible reactions with proteases have been reported by Abderhalden (1914) from autolyzing tissues. Taylor (1909), found that a protein—" plastein " —could be formed from albuminose and proteolytic enzymes. The reversible reaction of starch to sugar at low temperatures and sugar to starch at high temperatures is a well known reaction that takes place in potato storage. The cold hardy mechanism of these larvæ studied may, in part, be due to enzyme action which transforms large protein molecules into smaller amino-acids. The larger number of osmotically active units thus formed would lower the freezing point.

Periodicity to cold hardiness is not as marked in the Japanese beetle as it is in some of the insects that are exposed to extremes of low temperature. Larvæ of the Japanese beetle live close enough to the surface of the ground to experience some seasonal change. During the spring and fall they are in addition subjected to diurnal temperature change. Cold hardiness in the larvæ appears to be closely related to their environment. These organisms are somewhat seasonal in their resistance to low temperatures. This periodic cold hardiness is shown in Table I. Comparison with oak borers and aquatic insects is shown more fully in a previous article by the author (Payne, 1926). Although the larva stage is the only one which overwinters in this climate, it was thought that studies on the cold hardiness of the adults would yield valuable material for the comparison of a stage exposed to winter conditions and a stage not normally exposed. Adults captured in summer and frozen without previous conditioning were able to survive ice formation within their tissues and to survive temperatures as low as — 20° C. Since it was impossible to obtain enough blood from the adults to make a conductivity reading none were made.

A beginning was made on the study of cold hardiness of the Japanese beetle pupæ. From present observations the age of the pupæ and consequently the degree of hydrolysis they are undergoing determines cold hardiness.

No changes in blood pH were found to be associated with cold hardiness in healthy larvæ. The pH obtained from a series of blood samples is shown in Table V. In the early stages of wilt disease the pH was lower than in healthy larvæ. In the late stages of the disease the larvæ were in such condition that it was difficult to obtain blood by cutting off the feet.

TABLE V.

PH OF JAPANESE BEETLE LARVÆ BLOOD THIRD INSTAR.

Each reading is an average of 3.

Healthy.	With Wilt Disease.
6.5	5.8
6.78	5.7
6.92	5.56
6.94	6.
6.5	5.91
7.16	5.82
7.18	5.83
6.66	5.97
6.77	6.1
7.1	5.84
7.17	5.92
7.	5.96
6.54	5.98
6.66	5.96
6.82	5.94
7.	5.9
6.35	5.84
6.51	5.97
7.1	5.95
7.1	

The respiratory quotient tends to be high in both cold hardy and non-cold hardy specimens, ranging from .67 to .72. The respiratory quotient of starving larvæ tended to be higher than well fed larvæ regardless of the temperature at which they were kept. The respiratory rate in larvæ in which cold hardiness had been induced was much lower than in the non-cold hardy individuals. Associated with the low respiratory rate of hibernating forms was the slight change in body weight occurring over a period of several months, as shown in Fig. 1.

COLD HARDINESS TO THE QUANTITY FACTOR OF LOW TEMPERATURE.

Both the second and the third instars of the Japanese beetle larva are cold hardy to the quantity factor of low temperature except directly after molting or when the digestive tract is clear. Larvæ are markedly adapted to withstand long periods of low temperature. At the present writing there are still ten larvæ alive of one hundred which were placed at + 10° C. on December 6, 1925. These larvæ have now been kept over two years below their developmental temperature. Similar lots have been kept from six to twelve months at + 10° C. Graphs showing the number of larvæ surviving plotted against time in months in these experiments are shown in Fig. 3.

The relationship between survival for long periods at low temperatures and cold hardiness to the intensity factor of low temperature is shown in Table VI. The two types of cold hardiness appear to be inversely related after a certain point has been reached. This decrease in cold hardiness to the intensity factor of low temperature cannot be interpreted as a simple loss in vitality since larvæ kept at low temperatures are able to complete their development when placed at room temperature with no higher death rate than larvæ maintained at room temperature.

TABLE VI.

SURVIVAL AFTER FREEZING OF JAPANESE BEETLE LARVÆ.

Kept at constant temperature of + 10° C. for varying periods of time.

Length of Time Kept at + 10° C.	Number Frozen.	Number Survived.	% Survived.
2 weeks	1,450	1,426	98.34
4 weeks	1,400	1,078	77
8 weeks	1,000	645	64.5
3 months	500	290	58
6 months	200	48	24

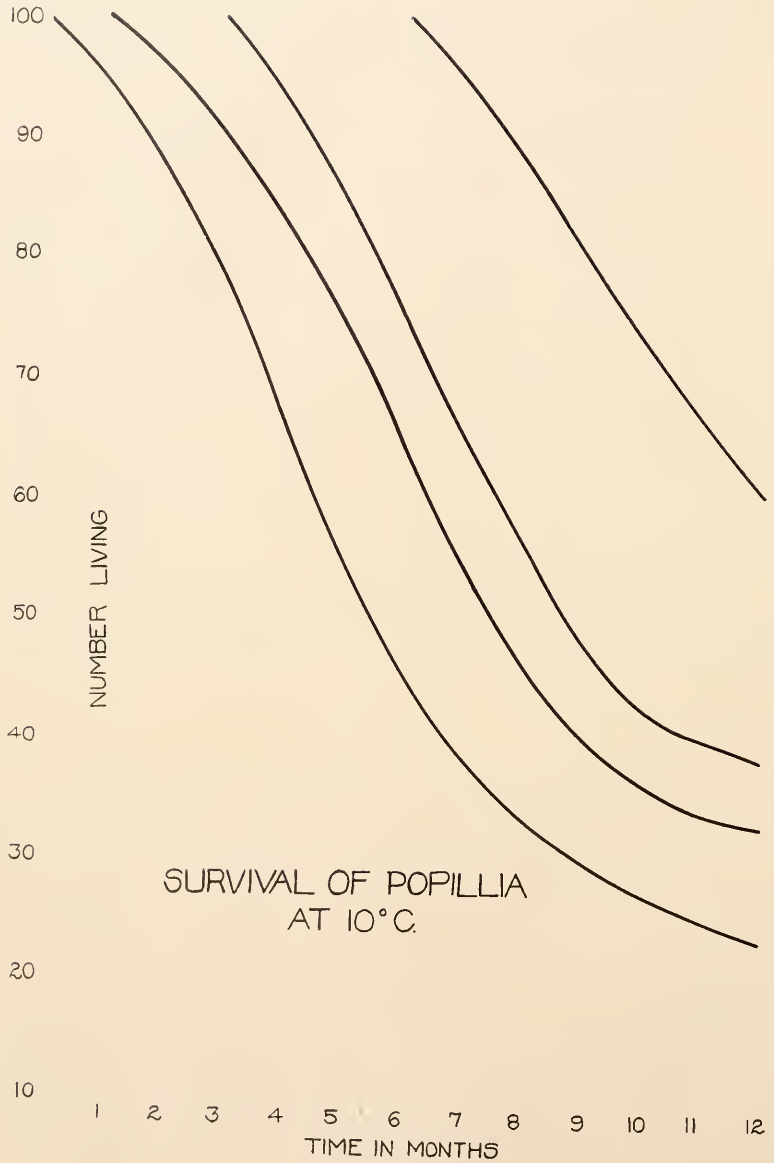
100
90
80
70
60
50
40
30
20
10
NUMBER LIVING
SURVIVAL OF POPILLIA
AT 10° C.
1
2
3
4
5
6
7
8
9
10
11
12
TIME IN MONTHS

FIG. 3.

Long periods of dormancy accelerate development when the larvæ kept at low temperatures are raised to developmental temperatures. Blood conductivity at first rises, then falls after two or more months when larvæ are placed at or below + 10° C.

The effect of rapid alternation between high and low temperatures on cold hardiness was tried with one hundred third instar larvæ. Temperatures of 0° C. and + 30° C. were alternated every twenty-four hours for one month. Neither of these temperatures is fatal. As controls one hundred larvæ were kept at 0° C. and one hundred at + 30° C. None died at + 30° C. Those alternated between + 30° C. and 0° C. died more rapidly than those kept at 0° C. Results of these experiments are shown in Fig. 4. In larvæ which had been exposed to wilt disease alternating temperature had no effect on length of life. None of these larvæ lived longer than ten days except when they were kept at or below 0° C. Healthy larvæ were considered exposed when they had been bitten by larvæ having wilt disease.

The respiratory quotient of larvæ cold hardy to the quantity factor of cold was somewhat variable but not connected to length of survival at low temperatures. In larvæ with clear digestive tracts it tended to become lower. In larvæ kept at + 10° C. it ranged from .69 to .73, or slightly higher than in larvæ cold hardy to the intensity factor of low temperature. In larvæ with clear digestive tracts low respiratory quotients were associated with lack of cold hardiness.

Low respiratory rate is associated with cold hardiness to the quantity factor of low temperature. Changes in body weight, as has been stated before, were very small with larvæ kept for long periods of time at + 10° C. These changes occurring in different states of nutrition and under varying temperature and humidity conditions are shown in Fig. 1.

Dehydration of larvæ is associated with cold hardiness to the quantity factor of low temperature as well as to the intensity factor of low temperature. Dehydration beyond two thirds of the body weight decreases cold hardiness to the quantity factor of low temperature. Over dehydrated larvæ lived but one day at 20° C. and not more than three days at + 10° C. or not more than four days at 0° C. Dehydrated larvæ have been kept for

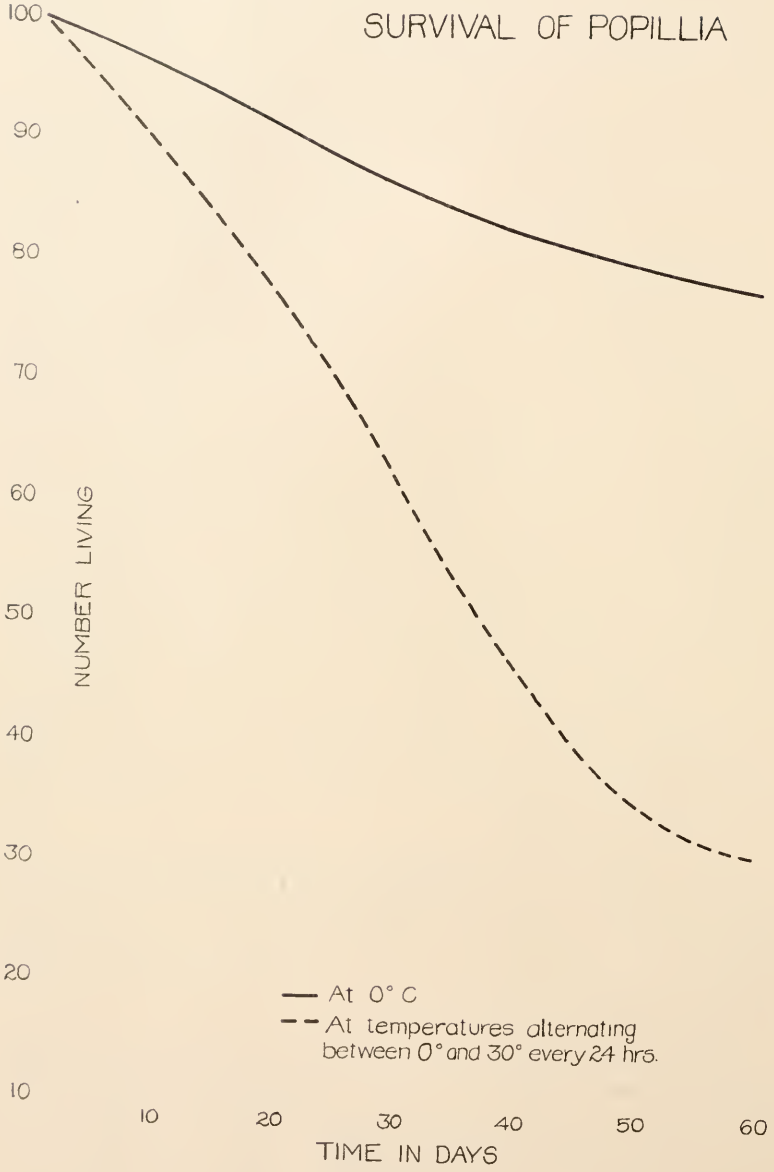
SURVIVAL OF POPILLIA
100
90
80
70
60
50
40
30
20
10
NUMBER LIVING
10
20
30
40
50
60
TIME IN DAYS
— At 0° C
-- At temperatures alternating between 0° and 30° every 24 hrs.

FIG. 4.

one year at + 10° C. The experiment has not been continued long enough to determine whether or not dehydration increases the cold hardiness to the quantity factor of low temperature. Untreated larvæ are able to live two years or more below their developmental temperature. Dehydrated larvæ show very nearly the same death rate as undehydrated ones.

LITERATURE.

Since the literature pertaining to cold hardiness has been recently brought together it seems hardly necessary to make a detailed list and discussion of it. Robinson (1927) has discussed and given experimental data on water binding capacity as a factor in cold hardiness. Robinson (1926), and the author (1926) have summarized the literature. Hibernation in regard to both its ecology and physiology has been recently treated by Fink (1925), Townsend (1926), and Holmquist (1926).

From a survey of the literature it would appear that no one factor is an adequate measure of cold hardiness. The development of a cold hardy from a non-cold hardy insect is a deep-seated physiological process which affects blood, and body fluids, respiratory rate and permeability. Nutritional state and environmental conditions also influence cold hardiness.

ACKNOWLEDGMENTS.

The facilities for this study of cold hardiness were furnished by the Zoölogical Laboratory of the University of Pennsylvania. To Dr. J. H. Bodine, of the Department of Zoölogy, I owe thanks for many helpful suggestions, especially as regards apparatus and methods. To Dr. Henry Fox, of the Japanese Beetle Laboratory at Moorestown, New Jersey, I am indebted for most of the material used and for suggestions that have proved of service during the progress of the work.

SUMMARY.

1. Cold hardiness, both to the intensity factor and to the quantity factor of low temperature, were studied in the second and third instars of the Japanese beetle. Brief observations were made on pupæ and adults with regard to cold hardiness.

2. Japanese beetle larvæ are somewhat periodic in their cold hardiness to the intensity factor of low temperature, less so than the oak borers previously studied and more so than the aquatic insects.

3. Disease incidence, nutritional state, and degree of dehydration are associated with cold hardiness to the intensity factor of low temperature.

4. Development of cold hardiness to the quantity factor of low temperature is associated with loss of cold hardiness to the intensity factor except in extremely dehydrated individuals.

5. Marked permeability changes associated with enzyme action occur at the vital temperature minimum.

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PELAGIC DISSOCONCHS OF THE COMMON MUSSEL, *MYTILUS EDULIS*, WITH OBSERVATIONS ON THE BEHAVIOR OF THE LARVÆ OF ALLIED GENERA.[1]

THURLOW C. NELSON.

The larvæ of the common black mussel, *Mytilus edulis*, are abundant in plankton samples taken throughout most of the summer in all regions where this mollusc occurs, Stafford, '12. Recognition of the larva, as Stafford points out, is rendered easy owing to its horn yellow color, its relatively small umbones and its small depth. To these characteristics may be added the distinctive shape of the shell, being more pointed and of shorter height at the anterior end, Fig. 1. The size of the mature prodissoconch when ready to attach varies considerably as judged from measurements of the largest larvæ obtained from the plankton, and from measurements of the prodissoconch shell of newly attached dissoconchs. Measurements of ten of the largest larvæ found in the plankton in Maine waters are as follows, antero-posterior axis being given first.

360 × 338 μ,
360 × 320 μ,
376 × 344 μ,
350 × 312 μ,
336 × 304 μ,
368 × 320 μ,
336 × 304 μ,
360 × 312 μ,
360 × 320 μ, exclusive of dissoconch rim,
304 × 280 μ, exclusive of dissoconch rim.

The last two larvæ, although caught in the plankton, each bore a narrow rim of purple dissoconch shell, Jackson, '88. From these and from other measurements made upon *Mytilus* larvæ it appears that dissoconch shell may be secreted at any time after the larvæ attain a length between approximately 300 and 360 μ. Stafford, *l.c.*, gives the measurements of two mature prodissoconchs as 345 × 310 μ and 400 × 331 μ.

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[1] From the Zoölogical Laboratory of Rutgers University. Paper No. 14, New Jersey Oyster Investigation Laboratory.

The observations here reported were made during August, 1924 and August, 1927, in Frenchman Bay, Mt. Desert Island, Maine.[1] A collecting station some 100 meters from the laboratory point was marked with a buoy. With approximately 12 meters depth at mean low water this station lay in the full sweep of the tide through Frenchman Bay. Plankton samples of 25 liters were pumped here from various depths using a hose and oscillating clock pump, the majority of the samples being taken at the surface and at 7 meters depth. The *Mytilus* larvæ were collected by passing the water through a No. 18 treble extra heavy bolting cloth net, adding two or three drops of formalin to the catch and then drawing off the supernatant water bearing great quantities of the diatoms *Chætoceros* and *Rhizosolenia*. Table I. gives the numbers and stages of development of the mussel larvæ taken at the station, together with other data.

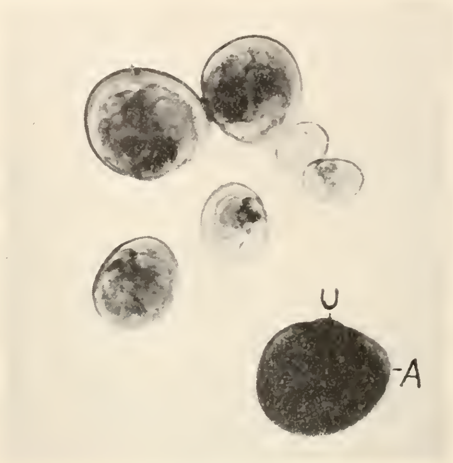

FIG. 1. Stages in the development of the prodissoconch larva of *Mytilus edulis: U,* umbones; *A,* anterior end.

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[1] It is a pleasure to acknowledge my indebtedness to the former Director, Professor Ulric Dahlgren, for the facilities given me at the Mount Desert Island Marine Biological Laboratory at Salisbury Cove, and for making early summer plankton catches for me.

Table I.

Water Conditions and Numbers of *Mytilus* Larvæ at Station off Laboratory Point, Frenchman Bay, Mt. Desert Island in 1924.

Date.	Time.	Tide.	Depth.	Temperature ° C.	Mytilus Larvæ in 25 Liters.	
					Prodissoconch.	Dissoconch.
Aug. 1.	—	—	Towing	—	—	1
5.	11:30 A.M.	2/3 flood	2 m.	10.6	Many	1
7.	3:00 P.M.	High	7 m.	11.0	262	
			0	13.3	753	
8.	3:45 P.M.	High	7 m.	10.9	336	
			0	17.1	71	
9.	11:30 A.M.	Low	7 m.	11.9	278	2
			0	15.6	5	
11.	10:15 A.M.	1/2 ebb	7 m.	13.7	63	
			0	14.3	6	
11.	10:30 A.M.	1/2 ebb	Towing	14.3	Many	8
12.	10:10 A.M.	1/3 ebb	7 m.	11.1	1,500	
			0	13.9	4	
13.	11:00 A.M.	1/6 ebb	7 m.	11.7	650	
			0	13.8	6	
13.	2:50 P.M.	3/4 ebb	7 m.	11.7	213	
			0	15.3	20	
14.	3:30 P.M.	1/6 flood	7 m.	12.3	240	1
			0	12.9	320	
15.	10:45 A.M.	High	7 m.	11.0	390	7
			0	12.9	1	
16.	11:20 A.M.	5/6 flood	7 m.	11.0	1	2
			0	12.9	177	
18.	10:50 A.M.	2/3 flood	7 m.	10.8	65	
			0	15.9	1	
19.	3:10 P.M.	High	7 m.	12.2	152	
			0	13.2	2	
20.	11:30 A.M.	1/3 flood	7 m.	11.9	56	
			0	12.7	2	
21.	2:50 P.M.	5/6 flood	7 m.	11.6	30	
			0	12.0	4	
22.	5:00 P.M.	High	7 m.	11.4	24	1
			0	14.7	5	
23.	3:10 P.M.	2/3 flood	7 m.	11.0	17	
			0	13.7	7	
25.	3:00 P.M.	1/3 flood	7 m.	11.7	40	1
			0	13.1	10	
27.	3:45 P.M.	1/6 flood	7 m.	12.3	19	
			0	13.9	7	

Pelagic Dissoconchs of *Mytilus*.

(*a*) *Buoyancy through Gas Secretion.*

In the tow sample taken by Professor Dahlgren August 1 was found one *Mytilus* larvæ which bore the distinct rim of purple shell which marks the commencement of the dissoconch stage.

Since only one such mussel was found it was believed to have been accidentally introduced through the townet striking some object bearing attached mussels. Subsequent collections, however, revealed numerous dissoconchs up to 941 μ in length freely floating about at various depths up to 7 meters. A 25 liter sample pumped August 13, from the surface, 20 cm. from a *Mytilus*-covered pile, yielded 200 *Mytilus* larvæ from mature prodissoconchs to advanced dissoconchs over 900 μ in length. A similar sample pumped August 15 from a depth of 7 meters at the collecting station, more than 100 meters from the nearest mussel beds, gave 390 prodissoconch *Mytilus* and 7 dissoconchs which ranged in length from 445 to 784 μ.

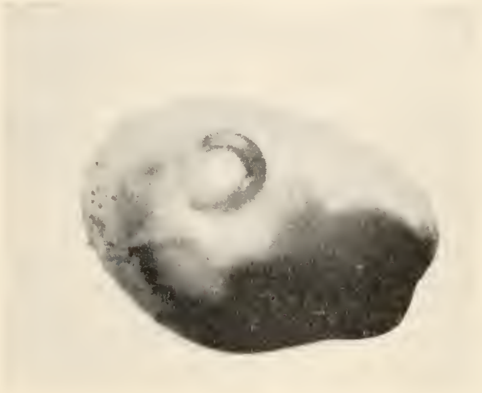


Fig. 2. Pelagic dissoconch of *Mytilus edulis* approximately .8 mm. in length, bearing a large bubble of secreted gas within the branchial chamber. This specimen came from a depth of 7 meters.

The presence of well-developed dissoconchs floating freely in the water at once raises the question of the means by which this is effected in the absence of the swimming organ or velum of the prodissoconch. When brought to the laboratory for examination these dissoconchs were found to be identical with others removed from sea weeds, save for the presence of a large clear space in the posterior portion of the pallial cavity. Believing that some change might have occurred in the molluscs even during the fifteen minutes to half an hour which elapsed between their capture and subsequent examination in the laboratory, a binoular was taken in the boat and the young mussels were examined immediately after their capture. The result is shown in Fig. 2. A large bubble was found to occupy the posterior part of the pallial cavity, its buoyancy causing the young bivalve to hang suspended in the water umbone downward, with the posteroventral margin of the valves turned upward. On one occasion the bubble was seen to form through the coalescing of many minute bubbles, which, passing slowly down between the gill filaments, united to form a single large bubble. In several individuals two or three smaller bubbles were found. Where a single bubble was present its size was such as to cause a thinning of the mantle on either side and a forward displacement of the posterior gill filaments, thus accounting for the large clear space already noted in the posterior pallial cavity of the young *Mytilus* dissoconchs first taken.

Failure to observe the bubble in larvæ first brought to the laboratory was due to the fact that as soon as a *Mytilus* dissoconch comes in contact with any object the foot is rapidly extruded from between the valves and brought into contact with the surface. The extrusion of the foot, accompanied as it is by a separation of the valves and of the applied lobes of the mantle, results in the immediate escape of the bubble in nearly every instance.

The composition of the gas in the bubble was not determined owing to its small size and lack of adequate facilities for a microchemical test. The fact that it forms within the gills would indicate that it is mainly oxygen. The composition of gas secreted into the swim bladders of fishes renders this still more probable.

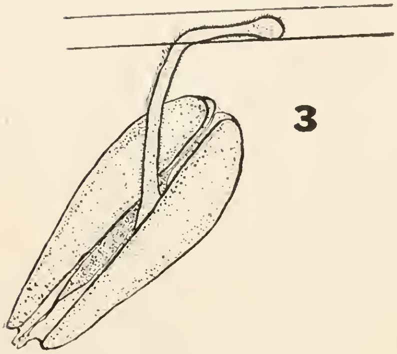
The possibility that the bubbles within the branchial chamber of these young *Mytilus* might have been air introduced accidentally during passage through the pump or while in the net, was tested in the following manner. The hose was disconnected from the pump and allowed to siphon water from a depth of 7 meters into the net held in the bottom of the boat. The stream entered the net under water and great care was taken not to agitate the net or to break the water surface. Dissoconchs of *Mytilus* collected in this way revealed the same large bubbles as before. Dissoconchs of *Mytilus* collected from sea weeds and violently shaken with a little sea water in a bottle failed to acquire any bubbles of air in the process; thus, with the above experiment, proving that the bubbles of gas were not accidentally introduced.

To determine the possible effects of pressure in bringing about gas secretion pieces of glass tubing 2 cm. long were cut and an early *Mytilus* dissoconch obtained from sea weeds was introduced into each. A piece of coarse bolting cloth was tied over each end of the tubes which were then fastened to a line at one meter intervals and suspended from a float at the collecting station. One string bore seven tubes which hung at depths of from two to eight meters. A second string was attached to a weight on the bottom with a float of sufficient size to hold the string vertically in the water, the lowest tube being at 11 meters depth at low water and approximately 15–16 meters at high water. When removed 48 hours later all of the bivalves were found to have attached by the byssus to the inside of the tubes or to the bolting cloth ends. When removed to a dish of sea water they crawled actively about with the foot. In no case was a bubble present. Either the stimulus to gas secretion is absent when the mussels are attached, or the frequent extrusion of the foot which occurs while the mussel is attached permits the escape of such gas as rapidly as it is formed.

(*b*) *Attachment to the Surface Film.*

If the surface of the water near a mussel bed or near a mussel covered piling be skimmed with a plankton net during the latter part of the breeding season, numerous dissoconchs will be found. They are most numerous as the rising tide first sweeps over the mussels and attached sea weeds. A microscopic examination of these dissoconchs shows that none contains a bubble, hence it is obvious that these young mussels must maintain themselves at the surface through means other than the gas secretion employed by larvæ at a depth. When placed in a dish of sea water such larval mussels exhibit great activity, gliding about upon the long, highly adhesive, ciliated foot as rapidly as a snail. Observations were made upon these young molluscs using a chamber 0.5 cm. wide made of two microscopic slides, filled with sea water and viewed horizontally through the binocular.

Once in contact with a solid object, such as the wall of the chamber, a rock, or a fragment of sea weed, the mussels exhibited a marked negative geotropism and climbed straight upward until the surface was reached. Here the distal one third to one tenth of the foot was extended in the surface film, Fig. 3, and with a quick contraction of the foot, aided apparently by contraction also of the pedal retractor muscles, the ventral margins of



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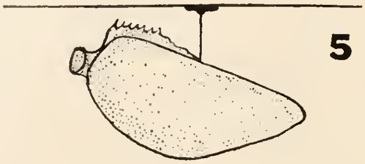

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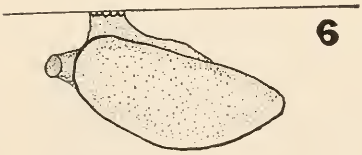

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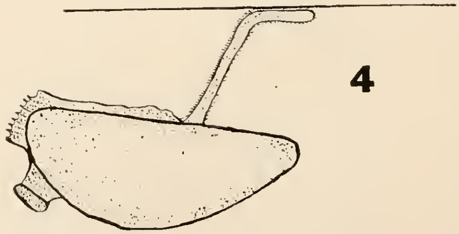

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FIG. 3. Ventral view of *Mytilus* dissoconch, 4 mm. long, hanging from the foot in the surface film, as seen from the side and partly from above.

FIG. 4. Lateral view of 4 mm. *Mytilus* dissoconch hanging from the surface film. The siphons are fully extended.

FIG. 5. Lateral view of 3 mm. *Mytilus* dissoconch hanging from byssus thread attached to holdfast secreted in the surface film. The foot, which was fully extended in the surface film while secretion of the holdfast was effected, has been wholly withdrawn between the valves.

FIG. 6. Lateral view of 3 mm. *Mytilus* dissoconch holding to the surface film with the aid of the tentacles of the incurrent siphon. The foot which serves to hold the mussel close to the film until the siphon is inserted therein, has been withdrawn between the valves.

the mantle were brought into contact with the surface film. While lying with the entire ventral margin of the body in contact with the surface film the byssus gland in a few seconds secreted onto the surface film a small holdfast similar in appearance to that which is laid down on rock or piling for the attachment of each byssus thread. A thread 1–2 mm. long serves to support the young mussel from this float and with foot withdrawn it may hang suspended indefinitely, Fig. 5. At times it thrashes about with the foot fully extended as though in search of some solid surface for attachment. When the foot strikes such an object the mussel glides quickly upon it, trailing the byssus thread behind or breaking it off. The "float" is not a buoyant structure, since when pushed beneath the surface film it rapidly sinks. It maintains its position in the surface film, supporting mussels up to 4 mm. in length, solely through surface tension.

A float and connecting thread are not always secreted when the young mussel reaches the surface. At times it supports itself solely by the distal end of the foot in the surface film, Fig. 4, after the manner described for the prodissoconch oyster larva, Nelson, '24a. With the aid of the numerous short cilia covering the foot the animal glides slowly along the surface film, rocking the body slowly from side to side and occasionally through a quick contraction of the proximal portion of the foot, bringing the entire ventral margin of the shell in contact with the surface film. This behavior will recall the familiar habit of pond snails of hanging from the entire foot spread out in the surface film.

A third mode of suspension from the surface consists in extending the tips of the tentacles of the incurrent siphon into the film and hanging from these, Fig. 6. This behavior, though seldom observed, serves to support the mussel quite as effectively as does the foot.

Such floating dissoconchs have never been found further than approximately 25 meters distance from mussel beds or mussel covered piling. Their abundance, 5 to 100 per 25 liters of water, close to such habitats indicates that young mussels frequently make use of this mode of transportation for covering short distances.

Examination with the low power binocular of several small tide pools close to the laboratory revealed numbers of *Mytilus* 3–4 mm. long moving actively over the rocks and barnacles while others were at the surface. With the incoming tide the latter are carried away and may eventually reach a place of attachment at a considerable distance.

Metamorphosis in Allied Lamellibranchs.

The water samples taken in Frenchman Bay contained in addition to the larvæ of *Mytilus edulis,* great numbers of the young of the soft clam, *Mya arenaria,* together with occasional specimens of the larvæ of *Venericardium,* of *Anomia* and possibly also of *Astarte.* During more than ten years study of the oyster larvæ of the New Jersey Coast I have become familiar also with the larvæ of *Mytilus recurvus* (the southern oyster mussel), *Venus mercenaria,* and *Teredo navalis.* In no instance have I ever observed gas secretion in any of these forms nor have I found pelagic dissoconchs. When the time for setting arrives the mature larvæ of all of the above species disappear from the water within 24 to 36 hours.

Reproduction and dispersal of marine pelecypod molluscs occur through the medium of pelagic veligers which are free-swimming for periods ranging from a few days in such incubatory forms as *Ostrea edulis* and *Teredo bartschi,* to approximately three weeks in *Mytilus edulis, Mya arenaria,* and in probably most of the marine bivalve molluscs which reproduce at temperatures below 20° C., Nelson, '28. Owing to the sessile or sedentary habits of the adult molluscs, the activities of the larvæ become of first importance in the dissemination and preservation of the species. Through the aid of the velum the larval bivalve, while unable to make progress against a current, can control its vertical distribution and thus secondarily may determine to a marked degree its horizontal distribution by tides and currents, Nelson, '22.

The rate of growth and of development during larval life is determined chiefly by the temperature. The long series of observations on the life history of the American oyster, *Ostrea virginica* Gmelin (J. Nelson, Stafford, Churchill, T. Nelson and others), indicates that at a given temperature the duration of the pelagic period is remarkably constant. With an average temperature of 23–24° C. the period from spawning to the attachment of the spat in New Jersey waters is 13 days. In Richmond Bay, Canada, J. Nelson, '17, found that at temperatures approximating 20° C. the minimum time required for oyster larvæ to mature was 17 days. Stafford, '13, considers three weeks to be the average time required to reach maturity in Canadian waters.

The close of the free-swimming period of pelecypod larvæ is determined apparently by internal developmental factors: when the time for "setting" arrives the larvæ must attach or die. Since the veligers during their pelagic existence have been distributed widely by currents it follows that for those which through chance happen to "fall upon good ground" there will be many more which through this same chance will "fall by the wayside" and be destroyed.

Observations of the oyster larva, T. Nelson, '22, '24, show that approximately 24 hours prior to attachment the young bivalve becomes positively stereotropic and that it may explore numerous surfaces with the aid of the foot before it finally attaches. Such behavior, while of the utmost importance in securing a favorable spot for attachment, is without avail if no substrata suitable for attachment are present. Little is known of the factors necessary to provide a favorable bedding ground for such burrowing species as *Mya* and *Venus*. Although attachment of young *Mya* by the byssus to sea weeds or other objects may occur, as shown by Ryder, '89, and by Kellogg, '99, it is pointed out by Belding, '12, that survival of both *Mya* and *Venus* depends largely upon the character of the mud and sand forming the surface layers of the bottom. All who have studied the habits of larval bivalves agree that the vicissitudes of larval life and subsequent attachment form one of the chief barriers to wide dispersal of the species.[1]

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[1] A survey of our present knowledge of the habits and life histories of both fresh-water and marine pelecypods shows that of all environmental influences the presence of a suitable substratum is the most important single factor limiting distribution. The following papers may be cited in this connection: fresh water mussels, Coker et al., '21; *Mya*, Belding, '09; *Pecten*, Belding, '10; *Venus*, Belding, '12; *Ostrea cucullata*, *O. angasi*, Roughley, '25; *Mytilus*, *Cardium*, *Saxidomus*, *Siliqua*, *Paphia*, and other genera of the Pacific Coast, Thompson, '13, and Weymouth, '20; *Ostrea*

Of the known genera of marine pelecypods, *Mytilus edulis* and *Teredo navalis* alone are circumpolar in their distribution over the shores of the northern hemisphere. General adaptability to changing conditions and the power to resist adverse surroundings together with relatively low spawning temperatures, Nelson, '28, have aided these two forms in attaining their present wide distribution. Transportation through attachment to vessels or to other floating wood has likewise aided in their dispersal, being for *Teredo* the only means by which any great distance could be covered. In the case of *Mytilus edulis,* however, the ability to bridge the period of metamorphosis while remaining pelagic must have been an important factor in securing the wide dispersal which this mollusc now enjoys; as well as a great aid in bringing to a suitable place of attachment a fair proportion of the larvæ produced each season. The largest of the pelagic dissoconchs found in Frenchman Bay was fully a month old, during which time it must have been transported over long distances by the tide. If during this period it had come in contact at any time with a solid object attachment could easily have been effected.

Summary.

At the close of the larval or prodissoconch stage the young of *Mytilus edulis* which fail to secure immediate attachment may remain pelagic through the secretion of gas into the mantle chamber.

Short distances may be covered through the aid of a holdfast secreted on the surface film or through holding the foot or the tentacles of the incurrent siphon in the surface film.

The ability to bridge over the critical period of metamorphosis while remaining pelagic has been an important factor in securing the present wide distribution of the black mussel.

Absence of a similar adaptation in the larvæ of other bivalves renders them still dependent largely upon chance in securing at the close of the pelagic period a proper substratum for attachment. This dependence upon the substratum is one of the chief barriers to the wide dispersal of the species.

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STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

VI. A FEMALE OF *CAMBARUS IMMUNIS* WITH OVIDUCTS ATTACHED TO OPENINGS OF SPERM DUCTS.

C. L. TURNER.

Zoölogical Laboratory, Northwestern University.

The specimen to be described was taken from Root River near Racine, Wisconsin, on July 10, 1924.

The presence of a normal annulus ventralis and of the usual rudimentary appendages upon the first abdominal segments mark it as a female but there are no oviducal pores at the bases of the third walking legs. On the other hand, a pair of openings appears at the bases of the fifth walking legs at the position ordi-

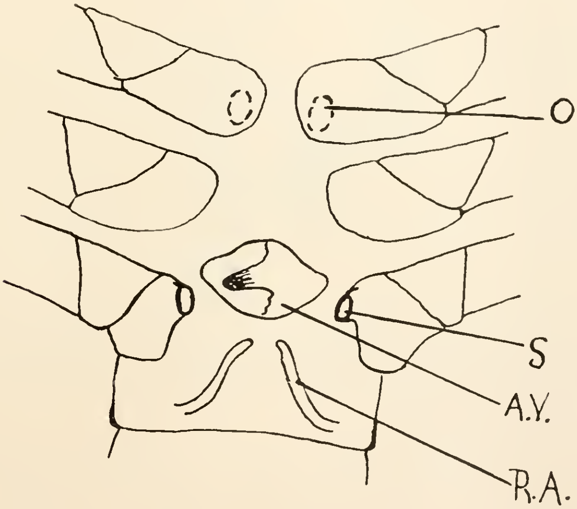

Fig. 1. Diagram illustrating external secondary sexual characters. *S*, openings of oviducts at base of fifth walking leg. *A.V.*, annulus ventralis. *R.A.*, rudimentary first abdominal appendages. *O*, position at base of third walking leg of oviducal pore in normal female.

narily occupied by the openings of the sperm ducts. No other male characters are found, however (Fig. 1).

Upon dissection the specimen was found to have a well-developed ovary and oviducts which were attached to the ovary at the usual point. In normal specimens the oviducts make their way laterally and ventrally and open through pores located at the bases of the third legs. In this case, however, the oviducts slope posteriorly as well and finally attach themselves to the openings at the bases of the fifth walking legs. A closer examination of the pores themselves shows that they are essentially the openings of sperm ducts in structure although they are considerably modified (Fig. 2).

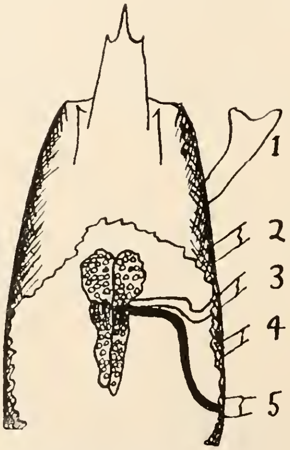

Fig. 2. Diagram illustrating internal relations of ovary, oviduct and walking legs. Position of oviduct in this specimen shown in solid black. Position of oviducts in normal female shown by lines. Positions of walking legs indicated by numerals.

The normal oviducal pore is oval in outline and has slightly raised and thickened rims, but the structure, as a whole, is not raised conspicuously above the surface of the shell. The greatest width of the oviducal pore in a specimen of this size is about one mm. with the length a little more. The plane of each pore is tilted a little toward the median line and a little toward the rear.

The normal openings of sperm ducts are much smaller and are extended by means of membranous projections. They open toward the median line and there is practically no deflection ventrally or posteriorly.

The openings of the specimen described here resemble the pores of sperm ducts in their general position, in the direction in which they open and in being projected somewhat by membranes. On the other hand, they are much larger than the openings of sperm ducts but smaller than normal oviducal pores and in shape resemble oviducal pores.

Discussion.

Several questions of interest arise in connection with this case but most of them can be treated only as speculations. (1) How does it happen that the oviducal pores are absent in a female animal? (2) How does it happen that the openings of sperm ducts are present in a female animal? (3) Is the duct to be interpreted as an oviduct which has become attached at its distal end to the opening of a sperm duct or as a vas deferens which has become attached at its proximal end to an ovary? (4) To what extent has there been a modification during its development of the pore of the sperm duct by virtue of its attachment to the oviduct? (5) To what extent does the oviduct possess the potentiality of shaping a structure to which it is not ordinarily attached (the opening of the sperm duct) in the direction of an organ to which it is usually attached (the oviducal pore)?

As regards the first and second questions, it is being repeatedly shown in crayfishes that the contemporary occurrence in an animal of the ovary or spermary and a fixed set of secondary characters is by no means necessary for normal functioning. Indeed it has been shown that in some localities the occurrence of a charter supposed to be fixed upon one sex occurs upon the other sex considerably more than half of the time. The lack of one or both oviducal pores in otherwise normal females has also been recorded. The absence of oviducal pores is not surprising, therefore, nor the presence of the openings of sperm ducts. The presence of the openings of sperm ducts in the absence of oviducal pores does not mean that these are mutually exclusive structures, for oviducal pores are sometimes found upon males normal with respect to the sperm duct openings as well as otherwise normal.

The tube connecting the pore at the base of the fifth walking legs and the ovary may be considered an oviduct for the following reasons. First, it is straight and shows none of the coils of the vas deferens. Second, it is thin walled and wide while the vas deferens is rather narrow and dense in texture. Third, it exhibits the same types and arrangement of tissues as a normal oviduct.

In *Cambarus* the only misplaced oviducal pores discovered have occurred on the second or the fourth walking legs of females and then only as supernumerary pores, the normal oviducal pores being present on the third walking leg as usual. It is assumed, therefore, that the pores of the fifth walking legs are the openings of sperm ducts and not of oviducts. The extent to which the pores depart from the features of the normal sperm duct is taken to represent the extent of the influence of the attachment of the pores to oviduct instead of sperm duct.

There is no evidence to show that the influence of the oviduct is required in embryology for the proper shaping of the oviducal pore. Rather to the contrary the oviducal pores are sometimes present and perfectly developed in the absence of oviducts. At the same time the resemblance of the pores of this specimen to oviducal pores must have been due to the influence of the oviduct to which they were attached. The case is roughly parallel to the results of the experiment in which an optic cup in a vertebrate embryo was transplanted under ectoderm in another part of the body and under the influence of the optic cup the ectoderm developed a lens although normally it would not have done so.

The condition described here is not to be confused with the normal condition found in some South American species belonging to the genus *Parastacus*. In these there are regularly both oviducts and sperm ducts but only one set is functional, that one being appropriate to the sex in which it occurs. The case described in this paper cannot be considered as a parallel, for here only one set of tubes is present and only one set of external pores.

STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

VII. REGENERATION OF ABERRANT SECONDARY SEXUAL CHARACTERS.

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It is rather generally conceded that intersexuality in insects has as a background either genetic disturbances or metabolic interferences due to parasitism. The evidence for the causes of intersexuality in the Crustacea, however, is by no means as distinct, due to the fact that it has not been possible to separate the question of the development of secondary sex characters from that of their control by hormones located in the gonads. It has been rather concisely demonstrated in the insects that secondary sex characters are independent of spermatic or ovarian hormones, whereas some of the evidence obtained in the Crustacea seems to indicate the possibility of such hormone control.

Among some six or seven causes which might be able to account for intersexuality among the decapods there is the repeated suggestion that some ambiguous or accidental event in embryology has been responsible. When in one locality eighty-six per cent. of all the females of *Cambarus virilis* and in another locality thirty-seven per cent. of *Cambarus propinquus* possess well-defined male characters, it would seem that no accidental event in embryology could account for this unusual occurrence. Rather, it would seem necessary to seek for some orderly and fixed influence. It occurred to the writer that the tendency to form aberrant structures might be tested for its duration beyond the embryonic stage by detaching the appendages bearing these structures and determining whether there would then be developed normal or aberrant structures. The accumulation of this body of data and the interpretation of it has been carried out with this inquiry in mind.

Regeneration of Normal Secondary Sex Characters.

When any appendage is lost in the crayfish, unless the crayfish is too old, a new appendage will grow in place of the old one and if the crayfish is young when the accident occurs the regenerated appendage will come to have almost completely the size and characteristics which the original appendage would have had. In an old specimen, however, there will not be sufficient time for the complete regeneration of the appendage before the crayfish dies.

Some of the external secondary structures which have to do with sex are either modified appendages or structures located upon the appendages. Such are the first and the second abdominal appendages which are modified for copulation in the male but are rudimentary in the female, the hooks located upon the third walking leg of the male, and the openings of the oviducts and sperm ducts upon the third and the fifth walking legs respectively. When one of these appendages is broken off so as to include one of the modified structures, the appendage will begin to regenerate, beginning with the first moult. At first it is juvenile in character and unmodified but eventually it becomes completely differentiated and contains the modified structure. The regenerated secondary sex characters never completely resemble the normal secondary sex characters. In the case of the hooks which are found upon the third walking legs, the regenerated ones are blunt and flatter than the original ones, but occurring as they do in a definite position they are easily recognizable.

Observations on the Regeneration of Aberrant Secondary Sex Characters.

Not more than thirty cases have been recorded in *Cambarus* of male-like modifications of the abdominal appendages in females and the possibility of finding cases in which the aberrant appendages have been injured and regenerated would be very remote. The occurrence of female structures upon males is also too rare to give any expectation of finding regenerated aberrant structures. The copulatory hooks upon the third legs have been selected, therefore, as the most likely structures for observation because their occurrence upon females furnishes the most common aberration.

In Lake Delavan (Wisconsin) eighty-six per cent. of the females of *Cambarus virilis* carry the copulatory hooks like those which occur upon the third legs of the males. It is as fully developed in the female as it is in the normal male and developes in ontogeny at the same stage. In the Menomonee River (Wisconsin) thirty-seven per cent. of the females of *Cambarus propinquus* also bear these hooks. These two localities were chosen as most likely to produce the desired specimens.

In the course of three summers collecting after several thousands of specimens had been examined, seven males and three females of *Cambarus virilis* from Lake Delavan were found, each of which had lost and regenerated one of the third legs. In each specimen the third leg had regenerated to a point where it was possible to determine whether or not a normal copulatory hook was being formed. Males, ranging from 56 to 88 mm. in length, had all somewhat imperfectly developed new hooks upon the regenerated legs (Fig. 1). These normal males with regenerated hooks were used as controls with which to compare the females which had likewise lost their third walking legs and had regenerated the third legs together with the hooks upon them. Of the females, two had well developed hooks upon the uninjured third legs and upon the regenerated third legs the hooks had reformed as in the males (Fig. 2). In the third female the uninjured third leg carried no hook and upon the regenerated third leg no hook had formed.

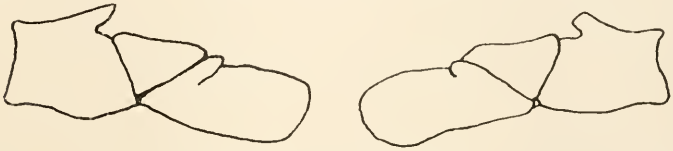

FIG. 1. Diagram illustrating three basal segments of third walking legs and copulatory hooks in male crayfish which has lost and regenerated a part of left leg containing copulatory hook. Regenerated copulatory hook is short and blunt.

In the Menomonee River, two specimens of *Cambarus propinquus* were found which could be used for this study. The first was a male in which the uninjured third leg was entirely normal and carried the usual hooks. The left third leg had been lost at an early stage and had regenerated. Upon it was the blunt type of hook usually found upon the regenerated third leg. The second was a female which also carried a hook upon the uninjured third leg and had also developed a blunt hook upon the regenerated third leg.

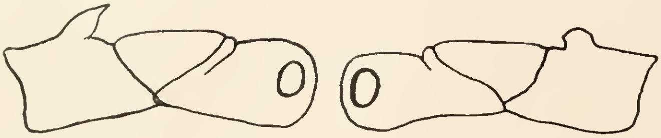

FIG. 2. Diagram illustrating three basal segments of third walking legs in an aberrant female which had lost and regenerated a part of the third walking leg. The copulatory hooks are aberrant and the left one has been regenerated.

EXPERIMENTS.

Young specimens of *Cambarus virilis*, about thirty-six mm. in length, were selected for experiment. They were taken from Lake Delavan on July 17. Eighty-two males in which the copulatory hooks on the third legs were visible were divided into two equal lots. One lot was used as a control and in the other, one of the third legs was detached in each specimen. Fifty-five females in which a copulatory hook was visible upon the third leg were divided into two lots and similarly operated upon or used as controls. All were kept in the laboratory under conditions as nearly natural as possible for ten months and about one fourth of the specimens survived. Six of these were males in which one third leg had been removed and upon the regenerated leg there had developed the copulatory hook. Seven were females from which one third leg had been removed. All such females had regenerated the third legs together with the copulatory hooks upon them. The hooks compared favorably with those regenerated by the males of approximately the same age.

CONCLUSIONS.

In all the cases cited above, whether observed in nature or experimentally produced, females bearing aberrant male hooks upon their third walking legs regenerated hooks whenever an injured leg had sufficiently developed. Some were one year of age and others were older. It is reasonable to state, therefore, that all the evidence, though meager, tends to show that whatever influence was present in the first place to produce this aberrant secondary sex character was also present and operative in the animal later during any regeneration period. The permanency of this influence during the life of an animal would seem to take it out of the classification of accidental or temporary embryonic agencies. When it is considered together with the fact that the same aberrancy is repeated in this crayfish population (observed for six years) it seems logical to give the influence a genetic status and to postulate that there has been a definite change within the germ cell.

REGENERATION OF *LUMBRICULUS* IN VARIOUS RINGER FLUIDS.

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INTRODUCTION.

In the course of work with Ringer solution on *Planaria,* J. W. Wilson ('26) has noticed that wound closure may be more or less completely suppressed in an isotonic solution. With the intention of making use of this peculiarity if it held true for *Lumbriculus,* I have experimented with various strengths of Ringer solution on this form. Finding various modifications of the usual method of wound closure and regeneration, I have made studies on the effects of various strengths of Ringer fluid on regeneration in this form. It is my purpose to report these at this time.

As a preliminary, the approximate osmotic pressure of the body fluids of this worm were determined in order that it might be possible to know something concerning the relative strengths of the internal fluids and the external solutions used. Adolph ('25, p. 332) concludes that we can "probably regard the maximum survival concentration for freshwater animals as a measure of the osmotic pressure of their body fluids." The maximum survival concentration of Ringer solution for *Lumbriculus* at the end of 24 hours (the arbitrary time adopted by Adolph, '25, for Phagocata) was found to be 0.147M. When corrected for ionization this gives a figure of 0.257M as compared with 0.215M for *Lumbricus,* as assumed by Adolph and Adolph ('25). Apparently a Ringer solution of between 0.14M and 0.15M concentration is approximately isosmotic with the body fluids of *Lumbriculus.*

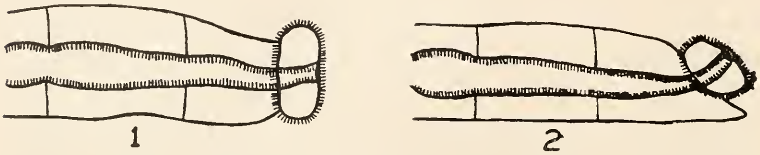
WOUND CLOSURE IN VARIOUS CONCENTRATIONS OF RINGER SOLUTION.

The usual method of wound closure in microdrilous annelids has been quite completely described (von Wagner, '00 and '06, Iwanow, '03 and Krecker, '10, among others), both from the gross and from the microscopical points of view. Briefly it takes place in somewhat the following manner: Immediately after a worm is cut the muscles of the body wall begin to contract. They continue to draw the cut edges of the hypodermis together until only a small opening is left. This aperture is then clogged by a plug of cells, many of which have been torn free by the cut. The wound is thus completely closed and the body fluids once more prevented from freely mixing with the solution in which the worm is cut. At the same time the intestine also contracts somewhat, closing over at the end and withdrawing slightly from the contracting body wall. This preliminary wound closure is completed in from 10 to 15 minutes after the cut is made.

The behavior in an isotonic solution is in distinct contrast to this usual behavior. The following description of what occurred in one series of observations might well apply to many cases which have been followed for considerable periods.

An individual is cut in a 0.14M Ringer solution, without anaesthetization, at 2:54 P.M. Both pieces move about quite rapidly at first but in 3 or 4 minutes they have quieted down to ordinary "crawling" movements, such as are commonly found in uninjured individuals. During this time there is a loss of some blood and a number of cells due to the fact that there is no semblance of contraction of the body wall. At 3:03 there is evidence of a protrusion of the gut beyond the end of the body wall. This protrusion of the gut gradually becomes more pronounced until a portion, perhaps a segment in length, extends beyond the plane of the cut at 3:10. At this time the cut end of the gut begins to show evidence of a rolling back upon itself. This process continues until at the end of an hour there is a well formed bulb of everted gut present at the cut end (Fig. 1). During this time there has been a gradual contraction of the body wall until it has reduced the diameter of the opening resulting from the cut to about two thirds of its original size. The gut in the course of its eversion has now come in contact with the body wall so that there is very little opportunity for interchange of materials between the body fluids and the external solution. This is the end of wound closure from the macroscopical point of view.

As a result of this process of "wound closure" there is present at the cut surface, at the end of 1 or 2 hours, a bulb of everted gut usually of almost as great diameter as that of the body. The ciliated portion of the gut cells are thus exposed to the outside solution, in which they continue to beat with apparently the usual rapidity.



Figs. 1 and 2. Bulbs of everted gut at the posterior end of pieces regenerating in 0.091M Ringer solution. Fig. 1 after 12 hours; Fig. 2 after 6 days.

In the case of slightly hypotonic solutions the resulting bulb of gut is usually smaller, in some instances not more than one fourth the diameter of the worm. The presence of these smaller bulbs is due, in part at least, to the fact that in these cases the gut does not protrude as far at first, so that when the eversion occurs there is only a short piece involved. A contributing factor is the slight pulling together of the body wall; this probably tends to hinder the protrusion of the gut and in addition reduces somewhat the diameter of the portion which does pass through the aperture.

Posterior Regeneration in Various Ringer Solutions.

If we assume that the maximum survival concentration at the end of a twenty-four-hour period is isotonic with the body fluids of an animal, it is difficult to keep individuals in an isotonic fluid for a very long period. One experiment may be cited to show what occurs when individuals are left for a long period in 0.147M Ringer, the solution being changed each day at the time of observation. Fifteen worms were put into such a solution and on the following day all were alive, with no ill effects apparent. On the second day, 2 had died and 3 others were clearly not far short of death. On the third day, 4 more were dead and 4 others were beginning to disintegrate at the posterior end. On the fifth day, only 5 were alive and 1 of these was beginning to disintegrate at the posterior end. Because of this high mortality in Ringer stronger than 0.14M, it has been found advisable to observe the regenerative processes in the latter concentration in which the majority of the regenerating head ends will live for a considerable period. Even in this strength many tails die in the course of 3 or 4 days.

In the case of heads, regenerating new tails, which have been cut and left in 0.14M Ringer, many individuals have bulbs very similar to those found after 2 or 3 hours, even at the end of a week. For example, of 12 heads put into such a solution, 9 had bulbs of everted gut, 1 had a short and very slender bud of regenerating tissue and 2 had died at the end of a week. This proportion of individuals with bulbs of gut holds true within fairly narrow limits in all experiments using 0.14M Ringer.

In light of these results obtained with a solution almost isotonic with the body fluids, it is of interest to determine the effect of slightly greater dilutions of Ringer solution. The number of bulbs of gut present on individuals "regenerating" in various strengths of Ringer solution for 7 days is given in Table I.

TABLE I.

NUMBER OF INDIVIDUALS WITH BULBS OF GUT IN DIFFERENT CONCENTRATIONS OF RINGER SOLUTION.

10 worms were cut in each concentration.
Figures in parenthesis indicate number which also show new tissue.

Molar conc. of Ringer.	Days of Regeneration.						
	1	2	3	4	5	6	7
0.011	0	0	0	0	0	0	0
0.023	0	0	0	0	0	0	0
0.034	0	0	0	0	0	0	0
0.045	3(1)	1(1)	1(1)	1(1)	1(1)	1(1)	1(1)
0.057	2(2)	0	0	0	0	0	0
0.068	3(1)	3(2)	1(1)	1(1)	1(1)	1(1)	1(1)
0.079	8(2)	8(4)	8(7)	8(8)	8(8)	8(8)	8(8)
0.091	7(3)	7(4)	7(7)	7(7)	7(7)	7(7)	7(7)
0.102	8(1)	8(5)	8(8)	8(8)	8(8)	8(8)	8(8)
0.113	10(0)	10(0)	10(1)	10(4)	10(10)	10(10)	10(10)
0.125	9(0)	9(0)	9(0)	9(3)	9(5)	9(6)	9(6)
0.136	10(0)	10(0)	10(0)	10(0)	10(0)	9(0) [1]	9(0)

[1] 1 dead on the sixth day.

After one day of regeneration all the bulbs of gut present are practically in line with the central longitudinal axis of the body (Fig. 1). The anal opening (not a true anal opening, for it is completely surrounded by gut which forms part of the outside wall in these cases) is, therefore, terminal. On the second day, however, those pieces in 0.102M and weaker solutions have bulbs tipped at an angle so that the opening is not exactly terminal (Fig. 2). This change in the position of the bulbs does not occur as soon in 0.113M and 0.125M; it does appear, however, usually in all individuals by the fifth or sixth day. But few individuals show any evidence of this tipping in 0.14M. In this respect the individuals cited in Table I. under 0.136M were exceptional, since none happened to show evidence of new tissue. The underlying cause of this slight shifting of the position of the bulb is apparently the growth of tissue on one side of the wound region and not on the other. The region in which growth occurs is found, on microscopical examination, to be always ventral.

There is, then, from the macroscopical point of view, a graded effect on regeneration in *Lumbriculus* produced by a series of various concentrations of Ringer solution. In an approximately isotonic solution the majority of individuals show little evidence of the production of new tissue; except in rare cases those individuals which do produce sufficient new tissue to make it apparent do so only on the ventral side. In weaker solutions new tissue is produced in practicaly all cases. In dilutions but slightly hypotonic (0.08M to 0.13M) this process is confined to the ventral region in most instances. In most cases where new tissue is produced throughout the wound region, no bulb of gut is present. Occasionally, however, a very small bulb may be found at the end of a regenerating bud which is 1 or 2 mm. long. In solutions below 0.08M, on the other hand, practically every individual produces new tissue throughout the wound region and bulbs of gut are rare.

Summary and Discussion.

In *Lumbriculus*, therefore, it would appear that an important factor in arousing the cells to regenerative activity is the dilution of the body fluids with water. When an individual is cut or broken into two or more pieces, there are open wounds through which the body fluids may flow out and water may enter. This entrance of water is perhaps increased by the "writhing" and "crawling" movements of pieces immediately after they are cut. These movements would also extend the region of dilution a slight distance from the wound. The author (Sayles, '27) has reported that for mesoderm and intestine regenerative activity involves between 10 and 12 segments from the wound region. In the case of the hypodermis, which is commonly bathed on the outside by a hypotonic solution, activation occurs only in a restricted region near the wound. This limited activity of the hypodermis is due perhaps to the fact that the diluted fluids come in contract with its inner surface underneath the muscles which are pulled away near the wound. Farther away than that, however, the diluted body fluids probably never reach the hypodermal cells through the relatively thick layer of muscle cells.

While the hypotonicity of the water to the body fluid seems to be an activating factor in *Lumbriculus,* too general conclusions cannot be drawn from such results. In other fresh water forms this factor may be found to be of importance but in marine animals regeneration can certainly take place in a medium which is presumably of the same osmotic pressure as their body fluids. In these animals, however, greater regenerative activity may occur in slightly diluted rather than in normal sea-water. This has been reported by Goldfarb ('07, p. 353) in the hydroid, *Eudendrium,* in which "the maximum number of polyps regenerated does not occur in normal sea-water but in solutions diluted with about 20 per cent. of tap-water."

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VARIATION OF HOOKS ON THE HIND WING OF THE HONEY BEE (*APIS MELLIFERA* L.).[1]

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Research Fellow of the International Education Board.

The present paper represents partial results of a series of investigations carried on by the author since 1924 in the field of biometry of the honey bee (See Alpatov, 1–10). The material for this work has been collected partly during the author's work in the Zoölogical Museum of the Moscow University, and partly (material on American bees) during the summer of 1927 in the apicultural laboratory of the Agricultural College, Cornell University. The definite calculations and the preparation of the manuscript have been completed during the winter 1927–28 in the Institute for Biological Research. The author is glad to express his deep indebtedness to Professor Koshewnikov (Moscow), Professor E. F. Phillips (Cornell University), and Professor Raymond Pearl for their interest and help. The author also appreciated very much the help given by beekeepers of Russia and U. S.A. in collecting bees from different parts of both countries. Profeessor E. F. Phillips has also been so kind as to show the author the manuscript of his unpublished paper.

In spite of the fact that the beekeeper's literature contains a tremendous number of observations on differences in bee races, a scientific basis of racial studies in bees is practically absent, especially in comparison with racial and genetical studies on other domestic animals. The cause of this lies chiefly in certain peculiarities which characterize the honey bee. Firstly, the bees being fecundated in air do not allow us to control the mating and therefore to conduct exact genetical experiments. Secondly, it is more difficult to study the characteristics of such small animals as the honey bee than those of domestic mammals and birds. Only quite recent progress in artificial insemination of the queen

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[1] From the Institute for Biological Research, Johns Hopkins University.

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(Watson, '20) gives us the hope of being able to overcome the first of these obstacles.

The present author believes that a careful investigation of variation should be made before any attempts to study the heredity of the honey bee. In this direction the present paper brings evidence of the importance of a certain characteristic, namely, the number of the hooks, characterizing different biological groups in the limits of the species *Apis mellifera* L. Thanks to the modern investigations mostly of Russian scientists (Koschewnikov, Chochlov, Michailov, Alpatov, Alpatov and Tjunin) two very important facts in the field of variation of the honey bee have been discovered.

The first of them is the geographical regularity in the variation. The changes in tongue length of the worker bee is the most striking fact in the geographical variation of the honey bee. We are able to say that for countries with native bee population each locality is characterized by a definite tongue-length of bees inhabiting the given locality. Moreover, the change from one locality to another is regular and gradual. A general rule can be established; the more to the south, the longer the tongue length. Other body characteristics also show some regularities in geographical variation (Alpatov, 8). The author of the present paper believes that it is perfectly justifiable to compare the different "races" of the honey bee with geographical subspecies of wild animals.

Family variation is the second important fact which every investigator in the field of variability of social insects has to bear in mind. It was shown by several investigators, Thomson, Bell and Pearson (23, 24), Warren (25), Arnoldi (12), Z. G. Palenitschko (20), Alpatov and Tjunin (1) and Alpatov (3, 4, 6, 9, 10), that the variation of single families is smaller than the variation of the whole population. Therefore, in establishing racial characteristics we have to collect our material from as many families as possible.

Turning our attention to the literature devoted to the special question of hook variation we find only a small number of papers dealing with that particular subject. Professor Koschewnikov (19) was the first who introduced the number of hooks in the taxonomy of the honey bee. E. B. Casteel and E. F. Phillips (14) without using biometrical methods, tried to solve the problem of comparative variability of drones and worker bees. Kellog's (17) data have also a very restricted value from the point of view of modern biometry. Wright, Lee and Pearson (27) then attempted, by recalculating Casteel's and Phillips' data, to draw some more definite conclusions. The most extensive work has been done by Bachmetjew (13). The conclusions of this author found just criticism in Koschewnokov's (18) and Raymond Pearl's (21) papers and need not be mentioned further. Fortunately, Bachmetjew published in his paper all his numerous countings (about 2,500 bees were examined). His data have been worked out biometrically by the author of the present paper, and published in Russian (4). Professor Phillips did the same in the paper which is now in press. In this paper Professor Phillips turns his attention mostly to the individual variation in

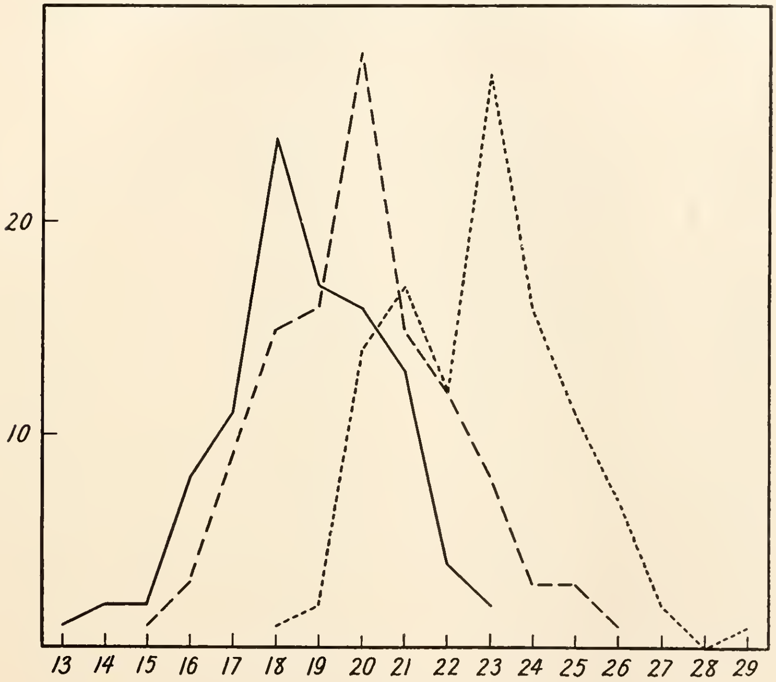

FIG. 1. Hook variation of 3 colonies of the Bulgarian drones.

Table I.

Constants of Variation Calculated for Drones of 10 Colonies of Bulgarian Bees Taken from Different Apiaries.
(Data from Bachmetjew.)

	Number of the Colony.									
	1	2	3	4	5	6	7	8	9	10
M	21.56 ± .11	21.59 ± .14	22.18 ± .13	22.75 ± .13	21.16 ± .16	22.69 ± .14	22.13 ± .13	21.53 ± .13	18.68 ± .13	20.05 ± .14
σ	1.645	1.831	1.622	2.001	2.755	2.053	1.665	1.802	1.959	2.151
C%	7.63 ± .37	8.48 ± .44	7.31 ± .41	8.80 ± .40	13.02 ± .56	9.05 ± .44	7.52 ± .36	8.37 ± .41	10.49 ± .51	10.73 ± .48
Number of cases	99	83	71	110	127	100	100	93	100	114

the honey bee, and on that account his conclusions do not parallel those of the present paper.

The number of bees examined by the author of the present paper exceed three thousand—a number which has never been reached by previous investigators.

Table I. shows us the variation of Bulgarian drones belonging to different colonies. It is evident that the difference between the averages are in many cases more than five times larger than their probable errors. Fig. 1 represents 3 variation curves of the 4th, 9th and 10th colonies, proving the conclusion just made. Table II. shows the same for worker bees. It can be seen that in the last case the differences are not so pronounced as in the case of the drones. Fig. 2 compared with Fig. 1 gives the same impression. If we consider the coefficients of variation we find that for the drones they vary in the limits 7.52–13.02 per cent.; for the worker bees 6.88–8.52 per cent. It is obvious that the average variation of worker bees of the colony is smaller than the variation of the drones.

Table II.

Constants for Workers of 5 Colonies of Bulgarian Bees (Data from Bachmetjew).

	Number of the Colony.				
	1	2	3	4	5
M	21.60 ± .10	21.01 ± .09	21.76 ± .10	21.91 ± .12	21.11 ± .10
σ	1.487	1.367	1.566	1.867	1.467
C%	6.88 ± .33	6.51 ± .31	7.20 ± .33	8.52 ± .41	6.95 ± .33
Number of cases	99	99	110	100	98

Are we justified in concluding that the drones are more variable than the worker bees? There is a certain weak point in such conclusions. We are not convinced that the method of collecting the material was safe enough to provide us with bees really representing the progeny of single queens—*i.e.,* members of one family. The proper way to get such a material would be to put a sealed brood in an incubator and collect the emerging bees. In collecting bees directly from the hive there is a danger of picking up bees belonging to the population of a neighbour hive. It is known that the bees and especially the drones sometimes penetrate into neighboring hives. The only way to avoid this difficulty is to calculate the coefficient of variation for the whole mass of bees. The results of such processes are shown in Table III. It can be seen that the variation of the worker bees belonging to

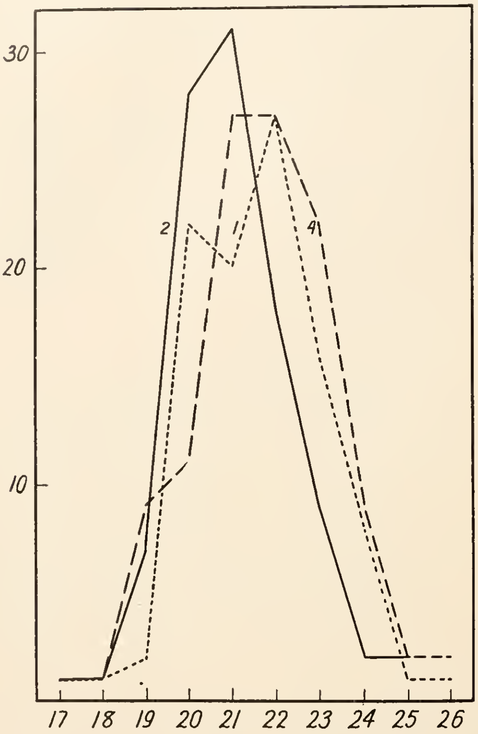

Fig. 2. Hook variation of 3 colonies of the Bulgarian worker bees.

Table III.

Bulgarian Bees in Different Groupings.

All Drones 1–10 Colonies.	Workers from 5 Colonies.	Queens from 10 localities in Bulgaria.	Drones from 1–5 Colonies.	Drones from 5–10 Colonies.
21.39 ± .05	21.49 ± .05	18.46 ± .11	21.82 ± .07	20.98 ± .07
2.352	1.586	1.892	2.157	2.438
11.00 ± .17	7.38 ± .16	10.25 ± .42	9.89 ± .42	11.62 ± .25
997	507	139	490	507

the 5 colonies is lower than the variation of the two groups of drones each representing members of 5 colonies. The coefficients of variation calculated from our original material on worker bees are also in general lower than 8 per cent. Even for 1000 worker bees from Middle Russia taken from 106 colonies the coefficient of variation is only 8.539 ± .129, as can be seen from Table X. We believe that the present material permits us the definite conclusion of a larger variability of drones in respect to the number of hooks.

Table III. contains also data on variation of hooks in queens. Firstly, it is evident that the average number of hooks is far lower than in the drones and workers, which have practically the same averages. This conclusion is given here in statistical form for the first time, although G. A. Koschevnikov has already given a few analogous data. In respect of the coefficient of variation the queens are nearer to the drones than to the workers. A very incomplete material collected in Table IV. shows that Middle Russian, German and American black and yellow queens have also a much lower average number of hooks than the worker bees of the corresponding races.

Table IV.

Number of Hooks of Drones and Queens from Different Localities.

	Drones.			Queens.		
	Moscow.	Kaluga (M. Russia).	N. Wodolaga (S. Russia).	Black-Ontario.	Italians.	Moscow and Darmstadt.
M	20.72	20.22 ± .20	20.83 ± .26	16.25	18.00	18.67 ± .22
C%	—	9.76 ± .69	12.00 ± .87	—	—	9.06 ± .83
No. of cases	25	45	48	8	10	27

It is interesting to note that among the bees the relations of castes in respect of variation differ from those found in other social insects. It was shown (Palenitschenko, 20) that among wasps, termites and ants, the workers are more variable than the sexual forms—males and females. The worker caste among bees is therefore an exceptionally constant and standardized group of individuals.

Already in an earlier paper (4) some evidence has been brought together to show that the bees of southern localities have a greater average number of hooks than the northern ones. In order to check that statement on a more solid basis, a special material has been collected from different parts of European Russia and the Caucasus. The map in Fig. 3 shows the localities which supplied a corresponding material. The plain of European Russia is populated by the black variety of *Apis mellifera* L—*A. mellifera mellifera* L. An introduction of foreign blood, mostly of Italian queens, was according to certain statistical studies a compara-

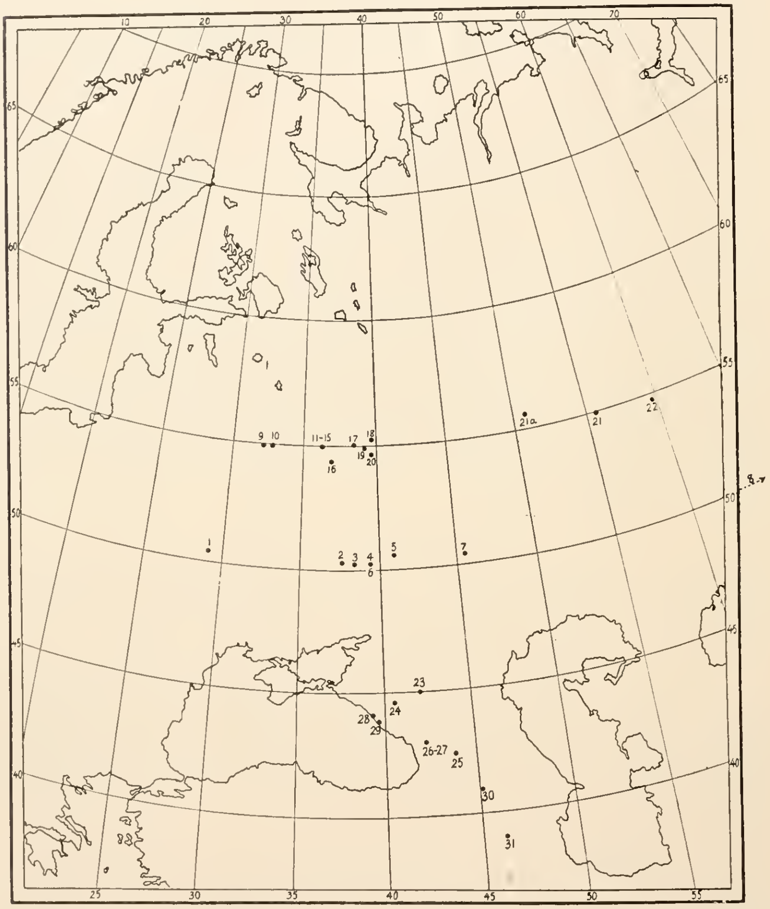
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FIG. 3. Map of European Russia and Caucasus. The figures correspond to localities where the material has been collected.

tively rare phenomenon and could probably not produce any significant influence on the whole mass of the bee population of Russia (the number of hives in Russia according to certain estimations runs over 5,000,000). Tables V. and VI show the frequency distribution for different localities, number of colonies and corresponding biometrical characteristics. Table X. gives summarized frequencies for the bees distributed in Middle Russia at the level of 55° of N. latitude and in South Russia, 50° N. latitude. The difference between the averages is 8.85 times larger than the probable error. We may conclude, therefore, with a high degree of certainty, that there is an increase in the average

Table V.

Frequency Distributions and Constants of the Number of Hooks of Bees from Middle Russia.

No. of Hooks.	Localities.													
	9	10	11	12	13	14	15	16	17–18	20	19	20a	21	22
29													1	
28													—	
27	1									1			—	
26	2				1					0		1	1	
25	1		1	1	0			1	2	3		2	2	
24	6		1	3	1	2	1	1	3	0		10	4	3
23	3	4	4	9	6	2	5	4	13	7	4	10	11	9
22	20	14	12	8	13	8	11	9	8	13	4	17	21	10
21	21	19	14	6	16	10	18	16	25	10	10	18	26	8
20	24	22	13	13	13	12	13	12	22	12	11	17	22	9
19	13	25	8	8	11	18	4	10	18	3	5	13	9	10
18	8	14	2	5	1	4	7	5	4	1	4	9	3	3
17	1	1	2	1		4	1	2	2		2	2		
16		1	2	1		1	1		3					
No. of cases	100	100	59	55	62	61	61	60	100	49	40	99	100	52
No. of colonies	7	10	6	6	6	6	6	6	18	6	4	10	9	6
M	20.86 ± .13	19.99 ± .10	20.56 ± .16	20.75 ± .18	20.92 ± .12	19.97 ± .15	20.56 ± .14	20.48 ± .14	20.58 ± .12	21.48 ± .16	20.28 ± .00	21.04 ± .15	21.27 ± .12	20.98 ± .16
C%	8.97 ± .43	7.27 ± .35	8.64 ± .54	9.45 ± .61	6.77 ± .41	8.51 ± .52	7.77 ± .47	8.04 ± .50	8.94 ± .43	7.97 ± .54	7.64 ± .58	9.11 ± .44	8.14 ± .39	8.08 ± .53

TABLE VI.

FREQUENCY DISTRIBUTION AND CONSTANTS OF THE NUMBER OF HOOKS OF BEES FROM SOUTH RUSSIA.

No. of Hooks.	Localities.						
	1	2–3	4	5	6	7	8
26	1		1	1	1		
25	3	1	0	2	2	3	
24	4	7	2	5	4	8	2
23	10	12	6	7	6	4	7
22	17	18	11	10	12	13	10
21	10	19	8	11	24	8	20
20	10	22	6	12	21	8	14
19	5	15	9	5	15	4	4
18	3	3	4	6	7	1	3
17	1	3	1	1		1	
No. of cases	64	100	48	60	92	50	60
No. of colonies	6	18	10	6	9	5	6
M	21.52 ± .16	20.94 ± .12	20.88 ± .18	21.13 ± .18	20.75 ± .12	21.68 ± .18	20.98 ± .12
C%	8.59 ± .51	8.29 ± .40	8.98 ± .62	9.53 ± .59	8.21 ± .41	8.63 ± .58	6.60 ± .41

number of hooks in the southern direction even between groups of bees in comparatively closely situated localities. Turning our attention to the Caucasus (Fig. 5) we must say that the situation here is more complicated than in the plain of European Russia. Zoögeographically, the Caucasus is divided into several sharply limited provinces, each of them with peculiarities in the composition and the origin of the organic life. The Caucasus bees are also not homogenous. The best characterized is the gray Caucasian mountain bee *Apis mellifera caucasica* Gorbatschev and the yellow Transcaucasian so-called Persian bee. This bee was first recognized as an independent species by Pallas; although he did describe the Caucasian bee he has never published his manuscript. The specimen with the original label is preserved in the Berlin Zoölogical Museum and was briefly described by

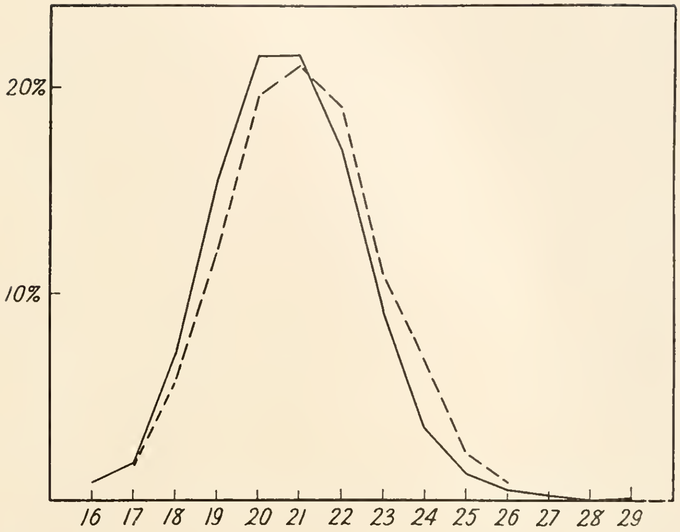

FIG. 4. The continuous curve represents the variation of hooks of bees from Middle Russia. The dotted, is based on material from South Russia. The frequencies are expressed in percents.

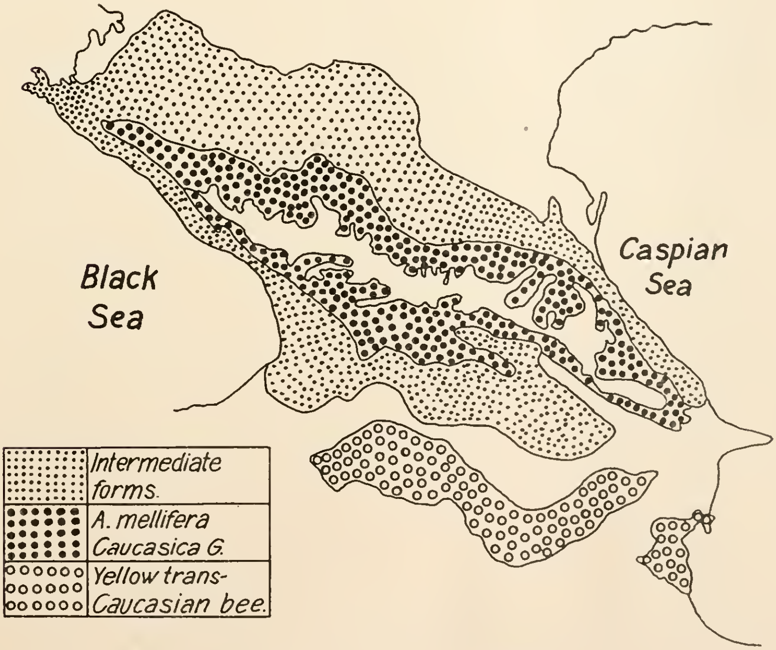
Black Sea
Caspian Sea
Intermediate forms.
A. mellifera Caucasica G.
Yellow trans-Caucasian bee.

FIG. 5. Map of Caucasus showing the distribution of fifteen variations of bees (after A. Gorbatschev).

Table VII.

Frequency Distributions and Constants of the Number of Hooks of Caucasian Bees.

No. of Hooks.	N. Caucasus.			Caucasus Mountains.					Abchasia.					Transcaucasia.		
	Stavropol 23.	Maikop 24.	Σ.	Tiflis 25.		Svanetia 26.	Kutais 27.	Σ.	I/28.	II/28.	III/28.	29.	Σ.	Migri 30.	Delidjan 31.	Σ.
27														1		1
26							1	1						1		1
25	1	2	3	1	3		0	4	1	2			3	0		0
24	2	1	3	0	3	2	2	7	0	1	2	4	7	2	3	5
23	10	8	18	6	2	10	2	20	9	4	9	8	30	4	7	11
22	24	19	43	13	5	10	9	37	6	9	9	10	34	8	14	22
21	40	24	64	9	6	6	9	30	9	10	19	17	55	4	8	12
20	43	30	73	12	4	4	4	24	8	8	12	13	41	8	6	14
19	24	12	36	7	1	2	5	15	9	5	5	8	27	1	1	2
18	6	3	9	2		2		4	3	2	2	5	12		1	1
17	5	1	6								2	0	2			
16	1		1									0	0			
15												0	0			
14												1	1			
No. of cases	156	100	256	50	24	36	32	142	45	41	60	66	212	29	40	69
No. of colonies	?	many	—	8	7	several	?	—	9	10	12	about 10	—	3	4	7
M	20.47 ±.11	20.79 ±.10	20.61 ±.06	20.94 ±.14	22.00 ±.24	21.61 ±.17	21.31 ±.19	21.33 ±.10	20.84 ±.17	21.10 ±.17	20.95 ±.14	20.82 ±.15	20.91 ±.08	21.79 ±.23	21.65 ±.14	21.71 ±.13
C%	7.31 ±.28	6.94 ±.33	7.14 ±.21	7.11 ±.48	7.87 ±.77	7.19 ±.57	7.49 ±.63	7.91 ±.32	8.14 ±.58	7.18 ±.58	7.42 ±.46	8.73 ±.52	7.93 ±.26	8.42 ±.75	6.16 ±.46	7.19 ±.41

TABLE VIII.

DIFFERENCES BETWEEN THE AVERAGE NUMBER OF HOOKS IN DIFFERENT GROUPS OF BEES.

		55°.	50°.	North Caucasus.	Abchasia.	Caucasus Mountains.	Trans-caucasia.	Italy.	American Italians.	American Black Bees.
		20.70 ± .04	21.08 ± .06	20.61 ± .06	20.91 ± .08	21.33 ± .10	21.71 ± .13	21.51 ± .08	21.00 ± .06	19.63 ± .09
55°..............	20.70 ± .04	0								
50°..............	21.08 ± .06	0.62 ± .07 R = 8.85	0							
North Caucasus....	20.61 ± .06	0.09 ± .07 R = 1.29	0.47 ± .08 R = 5.88	0						
Abchasia..........	20.91 ± .08	0.21 ± .09 R = 2.3	0.17 ± .10 R = 1.70	0.30 ± .10 R = 3.00	0					
Caucasus Mountains.......	21.33 ± .10	0.63 ± .11 R = 5.73	0.25 ± .12 R = 2.08	7.2 ± .12 R = 6.00	0.42 ± .13 R = 3.23	0				
Transcaucasia......	21.71 ± .13	1.7 ± .14 R = 7.22	0.63 ± .14 R = 4.5	1.10 ± .14 R = 7.86	0.80 ± .15 R = 5.33		0			
Italy.............	21.51 ± .08	0.81 ± .09 R = 9.00	0.43 ± .10 R = 4.3	9.0 ± .10 R = 9.0	0.60 ± .11 R = 5.45	0.18 ± .13 R = 1.38	0.20 ± .15 R = 1.33	0		
American Italians..	21.00 ± .06	0.30 ± .07 R = 4.29	0.08 ± .08 R = 1.00	0.39 ± .08 R = 4.87	0.09 ± .10 R = 0.09	0.33 ± .12 R = 2.75	0.71 ± .14 R = 5.07	0.51 ± .09 R = 5.67	0	
American black bees...........	19.63 ± .09	1.07 ± .10 R = 10.7	1.45 ± .11 R = 13.2	0.98 ± .11 R = 8.90	1.28 ± .12 R = 10.66	1.70 ± .13 R = 13.08	2.08 ± .16 R = 13.00	1.88 ± .12 R = 15.66	1.37 ± .11 R = 12.45	0

Gerstäcker (15). The author of the present article was able, thanks to the courtesy of the curator of the collection of Hymenoptera of the Berlin Zoölogical Museum, Professor Dr. H. Bischoff, to examine Pallas's specimen as well as his manuscripts. Pallas gives in his manuscript the following indication about the origin of his Caucasian yellow bee: "Ad Caucasum lecta, itemque ex Hyrcania transmissa fuit." The small size and pronounced yellow coloration of the specimens preserved in the Berlin Museum permit us to conclude that Pallas and Gerstäcker described under the name *Apis remipes,* the Transcaucasian Persian bee, but not the north Caucasian darker bees.

Some peculiarities—for instance a much longer tongue—distinguish *Apis mellifera remipes* Gerstäcker (not Pallas) from the Italian bee *Apis mellifera ligustica Sp.* It is therefore not correct to identify the *A. m. remipes* with the Italian bees (*Apis ligustica*) as it has been done by G. A. Koschewnikov. According to Gorbatschev (see the map in Fig. 5 taken from his article (16)) the prairies and hills of the northern Caucasus and the valleys of Transcaucasia are populated by a bee of intermediate type—hybrids in his interpretation. We united our material into four groups: (*a*) N. caucasus bees, (*b*) bees from four apiaries near the coast of the Black Sea—Abchasian, (*c*) gray Caucasian mountain bees (*A. mellifera caucasica* Gorb.) and (*d*) yellow Transcaucasian bees (*Apis mellifera remipes* Gerst.). Table VII. shows the frequency distributions and Table VIII. gives us material for estimating the importance of our differences. The *Apis m. caucasica* and *remipes* show a pronounced higher number of hooks than bees of South Russia. Of course such a comparatively limited number of colonies from N. Caucasus does not permit us to draw a perfectly definite conclusion. It is interesting to note that the gray Caucasian bees imported to the United States (see Table IX.) gave also a high average of the number of hooks.

Table IX. gives us some data on other European races of bees. The Italian bees from Italy are characterized by a high number of hooks. It can be seen that the progeny of Italian queens imported from Italy to N. Caucasus shows also a high number of hooks. The German black bees, according to our recalculations

TABLE IX.

BEES FROM DIFFERENT LOCALITIES IN EUROPE AND U. S. A.

No. of Hooks.	Caucasians from Colorado.	Italians from Italy.	Black Bees.				Italians from Caucasus.	German Black Bees (Armbruster).	Italians in U. S. A.							
			Florida.	N. Carolina.	Ontario.	Total.			Alabama 1.	Kentucky.	Alabama 2.	Ohio 1.	Ohio 2.	New York.	Ithaca, N. Y.	Pennsylvania.
26	1						1	0								
25	—	2					1	4			1			1	1	1
24	2	13		4		4	5	9	3	3	3	2	6	2	2	—
23	10	26		3		3	9	27	5	7	8	8	10	1	4	3
22	11	32	5	9	1	15	13	37	7	11	9	14	7	10	12	14
21	19	43	5	9	6	20	10	49	16	14	14	10	12	15	18	11
20	9	19	11	11	11	33	6	55	11	3	7	8	9	10	5	10
19	4	10	16	8	17	41	4	28	3	8	3	4	4	8	5	8
18	4	4	9	7	10	26	0	14	3	4	5	4	2	2	3	4
17		1	4	2	3	9	1	5	2					1	2	1
16				1	2	3		1								
No. of cases	60	150	50	54	50	154	50	230	50	50	50	50	50	50	52	52
No. of colonies	10	10	10	10	10	30	7	7	10	10	10	10	10	10	10	10
M	21.22 ±.14	21.51 ±.08	19.38 ±.13	20.37 ±.18	19.08 ±.12	19.63 ±.09	21.74 ±.21	20.84 ±.09	20.84 ±.16	21.06 ±.16	21.20 ±.15	21.16 ±.14	21.44 ±.16	20.72 ±.15	20.96 ±.16	20.57 ±.15
C%	7.46 ±.46	7.12 ±.28	7.07 ±.48	9.42 ±.61	6.78 ±.46	8.45 ±.32	7.51 ±.51	8.57 ±.27	7.87 ±.53	7.85 ±.53	7.40 ±.50	6.70 ±.46	7.64 ±.52	7.48 ±.50	8.02 ±.53	7.74 ±.51

TABLE X.

No. of Hooks.	1		2		3		4		5		6	
	♂ from 10 Colonies Bulgaria.		☿ from 5 Colonies Bulgaria.		☿ Middle Russian Bees.		☿ South Russian Bees.		☿ Italian Bees in U. S. A.		♂ from 1 Colony (Data from Phillips).	
	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.	Observed.	Calculated.
29.......	2	.81			1	0.20						
28.......	4	2.69			—	0.52						
27.......	9	9.34			2	1.40					2	1.64
26.......	21	24.01	5	2.14	5	3.99	4	2.32			8	6.91
25.......	52	51.34	12	9.68	13	11.60	11	9.41	4	4.83	21	21.52
24.......	84	91.22	34	36.12	35	32.93	32	27.80	21	17.60	54	49.74
23.......	149	134.73	81	81.13	91	84.15	52	59.74	46	46.82	82	85.15
22.......	154	165.41	112	122.08	168	171.39	91	93.65	84	82.77	104	107.93
21.......	183	168.76	121	122.68	217	243.76	100	106.93	110	100.00	98	101.40
20.......	142	143.07	97	82.60	215	222.94	93	88.87	63	82.57	74	70.61
19.......	87	100.92	33	37.21	156	134.34	57	53.86	43	46.65	38	36.40
18.......	61	59.05	8	11.24	70	59.20	27	23.76	27	18.00	15	13.93
17.......	31	28.75	4	2.26	18	21.95	7	7.65	6	4.75	3	3.94
16.......	11	11.63			9	7.60					1	0.83
15.......	4	3.91										
14.......	2	1.09										
13.......	1	0.25										
N.......	997	996.98	507	507.1	1,000	995.97	474	473.99	404	403.99	500	500.00
M.......	21.392 ± .050		21.485 ± .047		20.698 ± .038		21.082 ± .055		21.003 ± .054		21.708 ± .055	
σ.......	2.32525 ± .03511		1.57795 ± .03342		1.76733 ± .02665		1.77672 ± .03893		1.62084 ± .03846		1.82631 ± .03896	

TABLE X. (*Continued.*)

No. of Hooks.	1	2	3	4	5	6
	♂ from 10 Colonies Bulgaria.	☿ from 5 Colonies Bulgaria.	☿ Middle Russian Bees.	☿ South Russian Bees.	☿ Italian Bees in U. S. A.	♂ from 1 Colony (Data from Phillips).
C %......	10.870 ± .164[1]	7.344 ± .156	8.539 ± .129	8.428 ± .185	7.717 ± .183	8.413 ± .179
β_1.......	0.001585 ± .000723	0.019907 ± .010763	0.078324 ± .026122[2]	0.031206 ± .014291	−0.021689 ± .010207	0.000795 ± .000373
Ratio $\frac{\beta_1}{\text{P.E.}}$	2.192	1.850	2.998	2.184	2.124	2.131
β_2.......	3.247403 ± .146382	3.045287 ± .165798	4.194584 ± .104515[2]	2.719259 ± .107575	2.685580 ± .108751	2.817037 ± .110263
Ratio $\frac{\beta_2}{\text{P.E.}}$	1.690	0.273	11.429	2.610	2.89	1.708
K.......	0.002423	0.486326	0.027443	−0.037931	0.023560	−0.001621
Type....	Normal curve	?	VII	Normal curve	Normal curve	Normal curve
Goodness of fit...	.7326	.3131	.2602	.8775	.1819	.9977

[1] Calculated from the abbreviated formula like for all the other C. of V. Equation of the curve of the ☿ (Middle Russia, type VII)

$$y = 248.833 \quad 1 + \left(\frac{x^2}{21.935047}\right)^{-5.01133}.$$

[2] Calculated for normal curve of errors.

of Armbruster's data, give a number which corresponds to that of the Middle Russian ones.

Summing up now our whole material on European races we may say that there is much evidence for an assumption of a high number of hooks in southern races in comparison with northern ones. The Bulgarian group of bees also supports this conclusion. It would be interesting to test this rule on other castes of bee colonies. Unfortunately our material on drones from Russia is very small (see Table IV.), although it can be seen that the Middle Russian and even South Russian drones have a smaller average number of hooks than the Bulgarian ones. It would not be wise to draw any conclusions about the geographical differences in queens based on such a small number of cases. We have to add that Middle Russian and South Russian drones give the high degree of variation (C%) usual for drones.

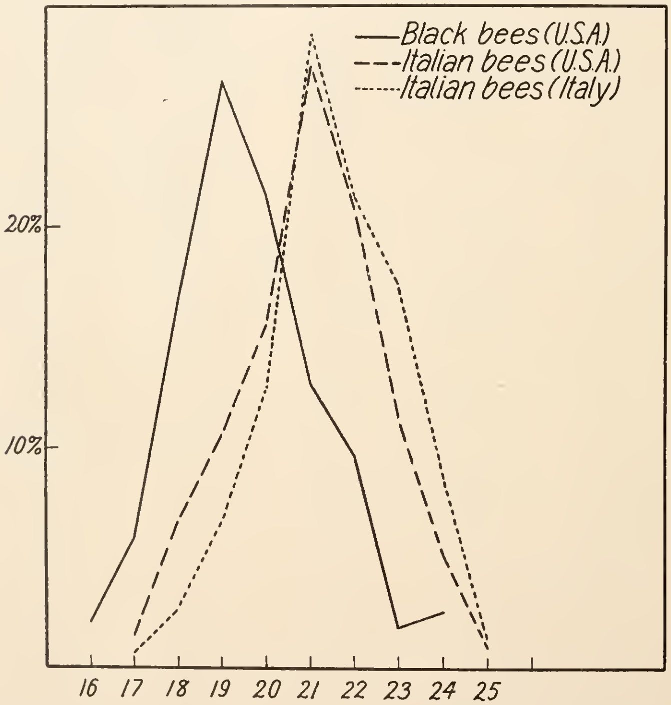

Fig. 6. Curves of variation of hooks. Continuous line—black bees in U. S. A.; dotted line—Italian bees from Italy; dash line—Italians in U. S. A.

It is well known that at the time of the discovery of the New World, America had no native bees. The first bees imported to this country came, according to historical data, from Holland and England and belonged to the common black bees *A. mellifera mellifera* L. About the middle of the last century the American beekeepers began to prefer for cultivation the yellow Italian bee, which is now the dominant race in this country. Thanks to the help of many beekeepers I have succeeded in examining, from a considerable number of apiaries, Italian bees of different degrees of development of yellow color as well as pure black bees. Tables IX. and X. show us the variation of bees acclimatized to the United States. Firstly, we have to note the great difference in the number of hooks of black and yellow American bees, secondly, a little lower number of hooks of Americanized Italian bees than that of true Italians reared either in Italy or from Italian queens imported directly from that country. This is illustrated by curves on Fig. 6. The very low average number of American black bees as compared with our material discussed

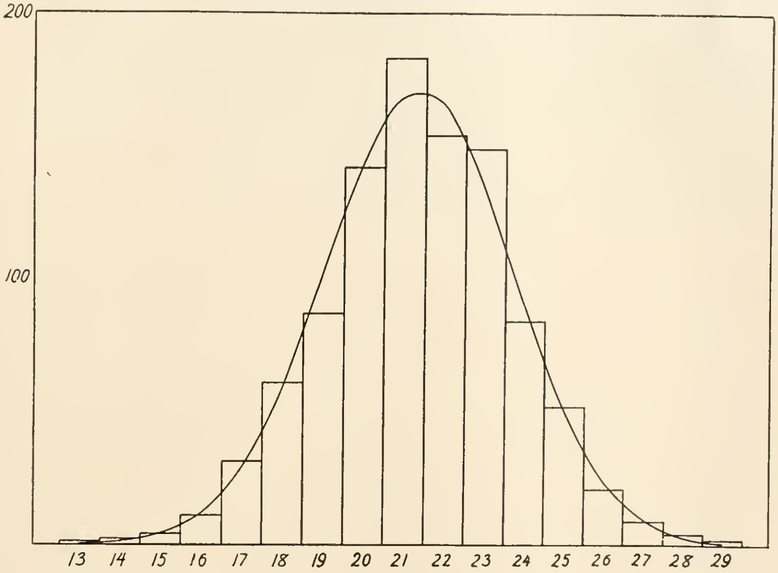

FIG. 7. Frequency polygon and fitted curve of the variation of the hooks of the Bulgarian drones.

above give us the right to suppose a general decrease of the number of hooks in the United States as compared with Europe, both in black and yellow bees. Further investigations need to be made with special attention to the problem of influence of acclimatization upon physical characteristics in the honey bee.

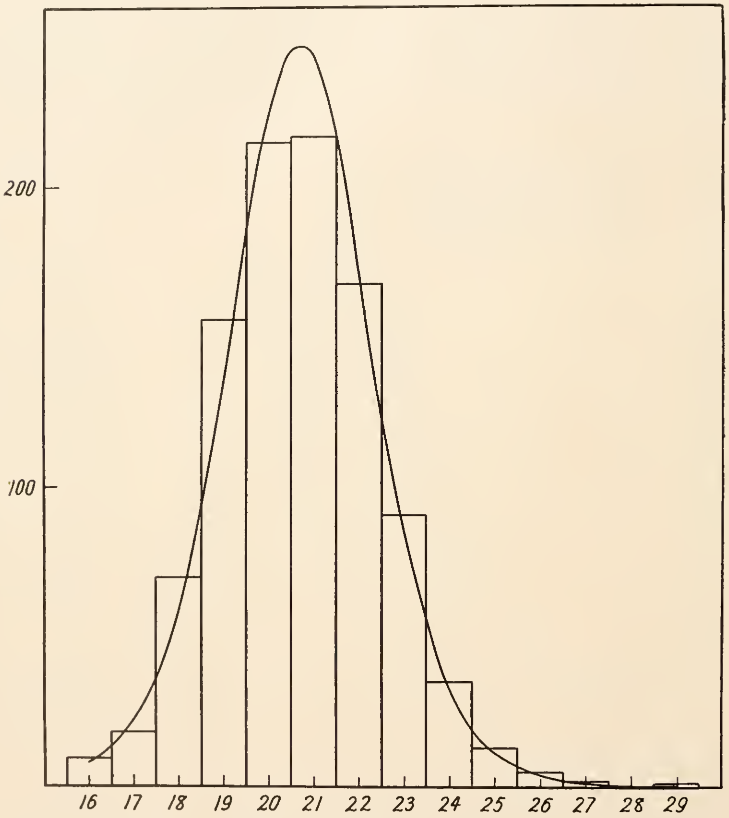

FIG. 8. Frequency polygon and fitted curve of variation of the worker bees from Middle Russia.

Our comparatively large material gave us the possibility of determining the character of the frequency distributions. The results are given in Table X. All distributions are symmetrical and only one shows a deviation from the normal distribution. A curve of type VII. was chosen to fit this distribution. The distribution for the Bulgarian worker bees being symmetrical and normal in regard to the β_2 gave a very high value of the criterion, which leads us to the curve of type IV. It was not possible to calculate the probable errors of the criterion, β_1 and β_2 being too close to those characterizing the normal curve of error. Therefore a normal curve was used for fitting. We used for calculating the ordinates of the normal curve from Pearson's "Tables for Statisticians and Biometricians." Figs. 7 and 8 show two of our curve fittings.

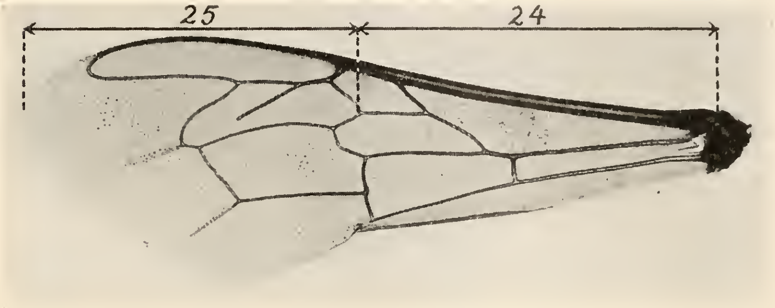

Fig. 9. Measurements on the wing. The wing shows the intercubitus vein not developed. (Microphotograph taken in the Art Department of the Institute for Biological Research, by Mr. Johansen.)

During the author's residence at Cornell University an attempt was made to study the influence of undernourishment of larvæ upon the characteristics of adult bees. The experiment consisted in putting the unsealed brood in an incubator running at 34.5° C. The brood was taken from a comb approximately one day before normal sealing. On the following day the cells situated in the neighborhood of the place from which a piece of comb had been taken the day before were already sealed by bees and also put in the incubator in order to provide us with control insects. Bees normally developed in hives were also collected from the frame of the hive which gave us material for the experiment. The pieces of comb with unsealed larvæ put in the incubator were covered with pieces of artificial comb foundation in substitution for the natural capping bees. The larvæ wove cocoons as usual and the emerging bees were collected in alcohol. The bees emerging from the unsealed brood evidently suffered from a certain underfeeding in comparison with control bees. Table XI. shows that the

TABLE XI.

CONSTANTS OF WING MEASUREMENTS OF CONTROL AND UNDERFED (IN LARVAL STAGE) BEES IN MM.

	Proximal Length of Wing (Meas. N 24).	Distal Length of Wing (Meas. N 25).	No. of Cases.
Experimental (underfed) bees.......	4.525 ± .021	4.192 ± .023	46
Control bees......................	4.696 ± .010	4.353 ± .012	31

experimental bees have a smaller size of wings than the controls. The characteristics have been measured, as is shown in Fig. 9. Table XII. shows the average number of hooks in three groups.

TABLE XII.

INFLUENCE OF UNDERFEEDING ON THE NUMBER OF HOOKS AND THE ABNORMAL VENATION.

Character.	M ± P.E.	C%±P.E.	Percentage of Wing with Abnormal Vein.	Number of Specimens.
1. Bees taken from the hive...	20.77 ± .16	7.34 ± .56	0.00 —	39
2. Bees reared in the incubator from brood normally fed..	20.60 ± .15	7.33 ± .52	4.44 ± 1.37	45
3. Bees reared in the incubator from underfed brood.....	19.71 ± .09	7.39 ± .33	19.82 ± 2.50	116
Diff. 1–3....................	1.06 ± .18 R = 5.89	—	19.82 ± 2.50 R = 7.93	
Diff. 2–3....................	0.89 ± .17 R = 5.24	—	15.38 ± 2.85 R = 5.40	

It can be seen that the underfed bees have a smaller number of hooks than the control bees reared from the sealed brood and taken directly from the hive. The same is expressed in graphical form on curves of the Fig. 10. The experimental bees showed a quite peculiar type of abnormality in the venation of the first pair of wings. The abnormality consists in the incomplete development of the second intercubitus vein. The percentage expression of this abnormality in our three groups shows that the abnormality occurs also in bees reared in the incubator from normally sealed broods but reaches a very high grade of development in undernourished bees. The abnormalities in insect wings have been many times the subject of careful morphological studies. Our experiment opens a way for studying this problem by means of the experimental method. It is worth while to note that in geographical races the southern bees, being usually smaller than the northern one (Alpatov, 8), at the same time show an increase in the number of hooks. Our experimental bees gave the contrary relation. Therefore it is not possible to explain the smaller number of hooks of the northern bees by the assumption of an underfeeding of larvæ.

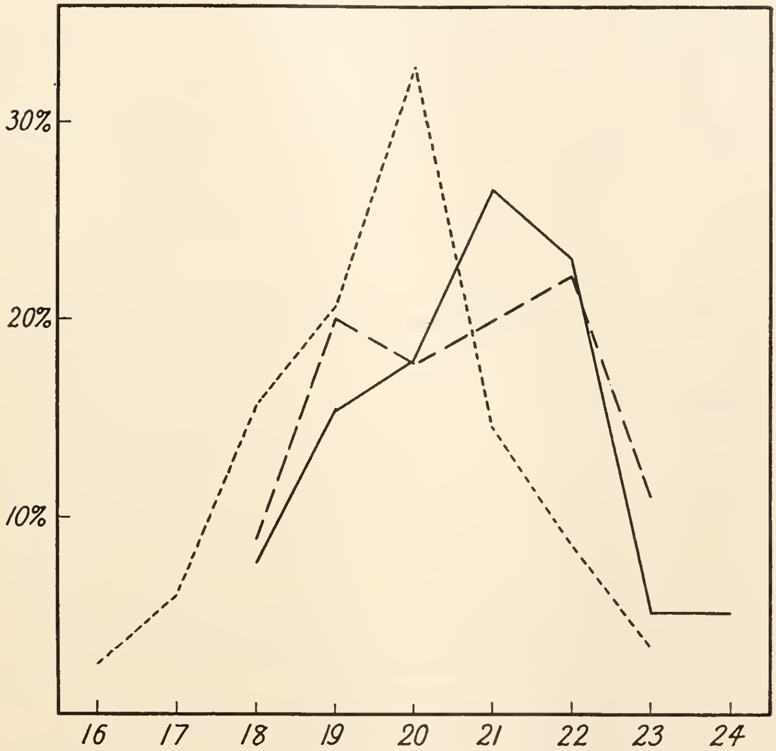

FIG. 10. Continuous line—bees from the hives which gave larvæ for the experiment with underfeeding. Dotted line—variation of hooks in the group of bees emerged from underfed larvæ. Dashed line—control bees developed from normally fed larvæ and emerged in the incubator together with underfed bees.

Summary.

The data presented in this paper show that the average number of hooks in the honey bee is a characteristic which is differently developed among single colonies, sexes, castes, and races. As a general rule the southern races have a large number of hooks in worker bees and probably in drones. The queens and drones are more variable in regard to this character than the worker bees. In this respect the relations differ from those in other social insects (ants, wasps and termites), where the asexual caste is the most variable. The experiment with underfeeding of larvæ showed a decrease of the average number of hooks and the producing of specimens with defective venation—incomplete second intercubitus vein.

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BIOLOGICAL BULLETIN

THE DEVELOPMENT OF THE SPERMATOZOÖN IN *CAVIA COBAYA.*[1]

MARY T. HARMAN AND FRANK P. ROOT.

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Introduction.

The development of the spermatozoön in the Mammalia has been observed in a number of forms but a detailed study has been made in only a few instances. Among the workers who have published observations on the development of the mammalian spermatozoön are: Lenhossek (1898), Meves (1898), Benda (1897, 1906), Korff (1902), Duesberg (1908, 1920), Jordan (1911), Oliver (1913), Stockard and Papanicolaou (1918), Gatenby and Woodger (1921). There is a general agreement in the plan of the development but many differences of opinion exist with reference to the detail. Many of these differences are significant not only from the development of the spermatozoön itself but also from their bearing upon other biological problems. Since mammals are bisexual and have not been known to reproduce parthenogenetically, the continuity of the different parts of the male germ cell is of as much significance as that of the female germ cell. The loss of a part of the nucleus or even a part of the cytoplasm in the process of transformation of the spermatid into a spermatozoön may affect the theory of the vehicle of the bearers of the hereditary characteristics.

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[1] Contribution from the Zoölogical Laboratory, Kansas State Agricultural College, No. 100.

Cavia cobaya has been used as a subject of investigation for the development of the spermatozoön as often as any other mammal and the work has been done in as much detail and yet there is a lack of agreement upon a number of points. All authors are agreed that the spermatid is a typical one, similar to that described for insects and other animals and that the mature spermatozoön is composed of at least three parts or regions, the head, the mid piece and the tail. Also a fourth region, the neck, has been described by many workers. What parts of the spermatid contribute to the formation of each of these regions, of what each region is composed and whether or not the entire cell is used in the formation of the spermatozoön are questions upon which there are significant differences of opinion.

In our study of the development of the spermatozoön of *Cavia cobaya* certain things have been impressed upon us as being decidedly different from the observations of other authors. Of these we shall mention five as the most outstanding: (1) Following the last maturation divisions the chromatin material goes through an abortive preparation for division before there is much change in the shape of the cell. (2) We have found no loss of cytoplasm or sloughing off as has been described by many authors. It is true that we find stages when the entire developing spermatozoön is smaller than in previous stages but this seems to be due to a condensation of the material rather than a sloughing off of any part of it. This will be discussed in some detail in the body of the paper. (3) We have not found in any stage a filament extending out from the cytoplasm. We have diligently looked for it because we were very anxious to see the nature of this development and at what particular time it was first evident. In all of our observations the axial filament tapers to a blunt point at the terminis. There is no naked end filament even in the fully formed spermatozoön. (4) The tail is made up of three segments which are not only shown by the morphological structure but also by the points of breaking as found in hundreds of broken specimens. (5) As was mentioned in our previous paper, the area of actively dividing cells are elliptical with the greatest diameter of the ellipse lengthwise of the tubule. Within this area the cells are generally in the same stage of development and only occasionally a stray cell is in some other stage.

It has not been our purpose to describe the origin of the cytoplasmic structures nor to say much about the confused nomenclature of the same. This has been only incidental to our purpose and we have discussed them only in so far as they contribute to the development of the spermatozoön. We have used much of the nomenclature of Bowen when it seemed applicable to our needs.

Material and Methods.

The material used is the same used in our previous paper (Harman and Root, 1926). In that paper will be found a detailed description of the fixing and staining of the material. All drawings have been made with the aid of a camera lucida and the magnifications are given in the description of the plates. With one exception, our drawings could be duplicated from hundreds of cells in our material. We make this statement to emphasize the fact that what we are showing is universal and not an exception which might be attributed to technique. The exception is the bent rod-shaped cytoplasmic inclusion in Fig. 7 which we have called a Golgi body.

(*a*) *Description of Material.*—We have begun with the changes which take place in the cell after the last maturation division has been completed. This is where we stopped in our last paper. For convenience of description these changes may be divided into three periods as follows: (1) The period with little change in the shape of the cell; (2) the period of elongation and (3) histogenesis of the elongated cell.

1. *The Period with Little Change in the Shape of the Cell.*—Significant changes take place both in the nucleus and the cytoplasm before there is much change in the shape of the cell. At the end of the last maturation division the chromatin passes through a typical telophase. It becomes finely granular and a definite nuclear membrane is formed. Following this there takes place what

we have chosen to call an abortive attempt to divide again. The chromatin forms into a close network having irregular clumps and the nuclear membrane nearly disappears, Fig. 1. Then the nucleus increases in size and the chromatin material is in a more nearly continuous spireme, Fig. 2. The chromatin clumps become more numerous and prominent. These changes continue until a compact unbroken spireme is formed, Fig. 3. Then there is an attempt to form chromosomes, Fig. 4. The chromatin knots are numerous and the spireme has been separated into irregular pieces which may be compared to chromosomes but which lack the smooth contour and the compact appearance of chromosomes. We have called these masses of chromatin material "chromatin knots." There remains some trace of the spireme but it is little more than a suggestion. Following this the chromatin knots become more granular and there is no further indication of a division of the cell, Figs. 5 and 6. Now the entire cell begins to contract and to become compact. At first this is more evident in the nucleus than in the cell body. The chromatin material becomes finely granular and only traces of the spireme are discernible. The entire nuclues occupies much less space, Figs. 7, 8, and 9.

While these changes have been taking place in the nucleus, changes have been occurring in the cytoplasm. A number of spherical bodies varying in size appear in the early spermatid. These are the Golgi bodies. There is a lack of constancy in the number and the size of these Golgi bodies. They are found in the periphery of the cell as well as near the nucleus. Sometimes they may indent the nuclear wall, Fig. 2. With Heidenhain's haematoxylin they are stained like chromatin which emphasizes their spherical form and distinguishes them from the surrounding cytoplasm in the early spermatids. They are finely granular like the surrounding cytoplasm but the granules are more closely compact than in the other parts of the cytoplasm. Each Golgi body has the appearance of a sphere surrounded by a halo.

An idiosome is always near the nucleus. In section it is crescentic in shape, with the concave side toward the nucleus, and stains like the surrounding cytoplasm from which it is distinguished by its more homogeneous structure. In older stages it comes to lie in contact with the nucleus then there is a more definite orientation in its position than that of the Golgi bodies and the nucleus. At least one Golgi body is always near the nucleus and at the same time near the idiosome, Figs. 1, 3, 4, and 5. Some of the Golgi bodies form a group near the nucleus on the side opposite to the idiosome.

The idiosome becomes closely applied to one side of the nucleus, Figs. 8 and 9. The idiosphere is in the concavity of the idiosome between it and the nucleus, *NE* in Figs. 8 and 9. The entire cell, both cytoplasm and nucleus, has become smaller and there is evidence of the beginning of the change in the shape of the cell.

2. *Period of Elongation.*—With the diminution of the volume of the cell there is the beginning of an elongation in the axis determined by the idiosome, on the one side, and the Golgi remnant on the other. This elongation occurs in the entire cell affecting the shape of both the cytoplasm and the nucleus, Figs. 10 to 22. During this time the cell is in intimate connection with the Sertoli cell which is at first small but later increases enormously in size. Fig. 20 illustrates a Sertoli cell with some of the associated spermatids in an elongated form. The part of the spermatid destined to become the head is directed toward the base of the Sertoli cell and the other part toward the lumen of the tubule. This is true regardless of the stage of development. When the spermatozoa are freed from the Sertoli cell they are not in a mixed up mass but are in bundles lying almost parallel, with most of the heads in the same direction. This could easily be accounted for by the fact that they have a definite orientation during their development. While the cell is elongating the idiosphere becomes embedded in the idiosome. The idiosphere stains more densely than the idiosome. Thus the idiosome has the appearance of having a core. The idiosome and the idiosphere form an elongate body pointed at its distal end and truncate at its proximal end, Figs. 13 to 17. Fig. 14 is a surface view while the other figures show the idiosphere surrounded by the idiosome. The idiosome and the idiosphere may now be called the acroblast.

The chromatin material in this stage has become finely granular and can scarcely be distinguished from the cytoplasm either by its staining reaction or by its structure. The nucleus elongates until it becomes cylindrical, Figs. 14 to 17. A thin coating of cytoplasm surrounds it and extends in the direction opposite to the acroblast. Later the nuclear material takes a position to one side of the cylindrical mass and the cytoplasm forms a flattened area to the other side extending from the acroblast to the other end of the cell, Figs. 18 and 19. In these figures the acroblast is becoming rounded and is beginning to take a position to the side of the nucleus instead of completely anterior to it as in the earlier stages. The nuclear material is beginning to become more condensed and is spread out over a wider surface. Posterior to the nucleus there are three fine thread-like filaments which spread into a somewhat fan-shaped mass in the surrounding cytoplasm. Associated with these filaments are two areas of cytoplasmic granules. One area is at the extremity of the filaments and the other area is near the base of the nucleus, Figs. 19 and 21. Following this stage, the cytoplasm which is transforming into the tail of the spermatozoön condenses rapidly and becomes very elongate.

3. *Histogenesis of the Elongate Cell.*—In the histogenesis of the elongate cell the three regions usually recognized in a mammalian spermatozoön begin to be evident. At first the nucleus and the acrosome which make up the head are much longer than they are wide and become cylindrical and somewhat enlarged at the free end. The nucleus is now at one side of the cytoplasmic acrosome and it does not extend entirely to the free end of the developing spermatozoön. The mid-piece which is occupied largely by the spiral filament in the adult spermatozoön becomes granular in regularly arranged clumps, *SF,* Fig. 23. This is the region which was occupied by the three thread-like filaments in Figs. 19, 21, and 22. One of the most noticeable changes is in the tail region. There is a very rapid condensation of the cytoplasm which was spread out in a fan-shaped mass to a tapering whip-like flagellum. The tail is composed of three segments. The first one is about as long as the mid-piece, the second one in the early stages is about the same length and the third or terminal one is a little longer than the combined length of the other two. It gradually tapers to a blunt point. We have not found in any stage of development any unsheathed terminal filament.

As differentiation progresses there is a greater difference between the sizes of segments one and two of the tail. The second segment elongates more than the first and tapers more as it increases in length. The segments are recognized by distinct markings and when the tails of the spermatozoa are broken off, the break is always at the union of two of these segments. Seldom is the tail broken from the head at the anterior part of the mid-piece and practically never is the tail broken off at the posterior part of the mid-piece. A few of the tails are broken at the end of the first segment. Most frequently the break is at the distal end of the first segment, less frequently between the second and the third segments. We never find the tail broken within any segment.

Figures 24, 25, and 26 are illustrations of a mature spermatozoön viewed from different positions. The acrosome forms a hood-shaped covering to one side and anterior to the nucleus. The head is broad from side to side, Figs. 24 and 25, but rather thin when seen from the edge, Fig. 26. The regularly arranged clumps of cytoplasm in the mid-piece, mentioned above, develop into a distinct spiral, with the coil always counter-clockwise from the anterior part of the mid-piece. The last two coils are almost rings and might be termed, annulus. There is no annulus separate from the spiral filament. As is shown in the drawings the coils are not always regular. They remind one of a spring that has been put at a tension and the rebound has not been the same in all regions of the spring. The first four coils of the spiral filament are inclosed by a thin bladder of cytoplasm.

Discussion.

In the transformation of the spermatid into the spermatozoön little attention has been given to the behavior of the chromatin material other than it finally becomes condensed into a more or less homogeneous mass which appears solid and is stained heavily with nuclear dyes. Meves (1899) has shown the nuclear material formed into clumps before there has been much change in the shape of the spermatid. Ballowitz (1891) has also this clumping of the chromatin material in his drawings. Neither author has discussed this change nor has mentioned further changes in the chromatin. They state that the nucleus forms the greater part of the head of the spermatozoön.

In one of the Hemiptera, Bowen (1920) says that "the head undergoes a characteristic change resulting in what appears to be a complete vacuolization of the chromatin lining. Then the chromatin collapses toward the axis of the head, etc."

We have shown that after the last maturation division the chromatin material passes through changes which are similar to those in a cell that is getting ready to divide until there is the breaking up of the chromatin material into clumps. A significant difference, however, between these changes and the changes previous to the maturation divisions is that there is no synezesis and no double thread. We raise the question whether these changes influence the behavior of the cytoplasm in the process of transformation and thus the attempt at division is aborted or whether the changes in the cytoplasm arrest the changes taking place in the nucleus.

The small size of the spermatozoön in comparison with the early spermatid is recognized by many authors. Some of this difference in size has been accounted for by a loss in cytoplasm. In the formation of the spermatozoön of vertebrates, Kölliker as early as 1856 and la Vallette St. George (1865) described the "sloughing off" of the cytoplasm. Later Biondi (1885), Benda (1897), Hermann (1889), and Neissing (1889 and 1896) agree that there is a loss in cytoplasm by a sloughing off. Meves and Ballowitz have shown cytoplasm loosely connected with the transforming tail part.

This difference in the size of the spermatozoön and the spermatid is recognized in the insects. Montgomery (1911) states that in *Euschistus* "no evidence was found for the casting off of any substance by the sperm."

In the formation of the spermatozoön in *Paratettix,* Harman (1915) did not find any loss of cytoplasm. The cytoplasm condensed around the axial filament but there was no indication of a sloughing off either in the appearance of the cell or the remains in the follicle.

In our material, the spermatozoön is greatly reduced in size during the process of transformation, but we have found no evidence in any region of a loss of material. We have shown, Figs. 24, 25 and 26, that a bladder-like structure of cytoplasm is present in the transformation but that this condenses around a portion of the middle piece and there is no evidence that it is sloughed off.

Most authors recognize that the greater part of the head of the spermatozoön is formed from the nucleus of the spermatid and furthermore, they recognize that this head is much smaller than the original nucleus. No one has described the loss of nuclear material. This agrees with our observations. We believe that this diminution in size is due to a condensation in which the material appears more compact than in earlier stages.

Meves (1899), Ballowitz (1891), and Duesberg (1910) show a thread-like filament extending out from the cytoplasm in the very early stages of development. Meves describes this filament as arising from one of the centrosomes which gives rise to the posterior nodule and this filament which in turn becomes the axial filament. He represents the distal end of this filament as remaining unsheathed and forming the terminal filament. We have found no unsheathed filament at any stage of development. We have shown, Figs. 18, 19, 21 and 22, three filamentous structures which lie deep in the cytoplasm. These filaments are spread out distally into a fan-shape. Associated with these structures are two areas of granules. We have not traced the detailed history of these granules but we have noted that they finally become inclosed in the cytoplasm which rapidly condenses and with the associated filaments form the tail of the spermatozoön. There is a gradual tapering of the tail to a blunt point. This tapering takes place in the axial filament as well as in the sheath which encloses it entirely to the distal end.

The tail is made up of three segments as we have shown in Figs. 23, 24, 25, and 26. Early in our study of the mature spermatozoön, among mutilated specimens we were impressed with the regularity of the lengths of the pieces of the tails. These lengths were quite constant whether the spermatozoa were in bundles, merely a few together or even if a single spermatozoön was broken. The pieces were in three different lengths which corresponded to the three segments of the tail. Measurements showed only a slight variation. It would seem that the tail is weaker at the points of junctions of the segments.

The transformation of the spermatid into a spermatozoön takes place in definite areas which are elliptical in shape. The greatest diameter of the ellipse is always lengthwise of the seminiferous tubule and the shortest diameter never exceeds two-thirds of the circumference of the tubule.

SUMMARY.

1. The transformation of the spermatid into a spermatozoön takes place while the spermatid is closely associated with a Sertoli cell and it does not become free in the lumen of the seminiferous tubule until the spermatozoön is matured.

2. In the early stages of transformation the cell goes through a growth period in which the entire cell gets larger and the chromatin material goes through an abortive preparation as if for division.

3. During the period of elongation there is a reduction in the volume of the cell and a rearrangement of its parts.

4. No "sloughing off" or loss of cytoplasm has been observed.

5. The head of the spermatozoön is composed of two parts, the head proper which arises from the nucleus and the head cap or acrosome which arises from the idiosome and the idiosphere.

6. There is a cytoplasmic bladder-like structure around the anterior part of the mid-piece.

7. The tail is composed of three segments terminating in definite nodes.

8. We find no indication of an unsheathed terminal filament either during the transformation or in the mature spermatozoön.

9. The tails of the spermatozoa are always toward the lumen of the seminiferous tubule.

10. The areas of transformation are elliptical in shape with the long axis of the ellipse corresponding to the length of the seminiferous tubule and the short diameter of the ellipse never exceeds two thirds the circumference of the tubule.

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EXPLANATION OF PLATES.

All the drawings were made with the aid of a camera lucida, a 1.9 oil-immersion objective and a number 6 compensating ocular at table level. Figure 20 was enlarged two diameters. All other drawings were enlarged four and one half diameters. The reproductions were reduced one half from the original.

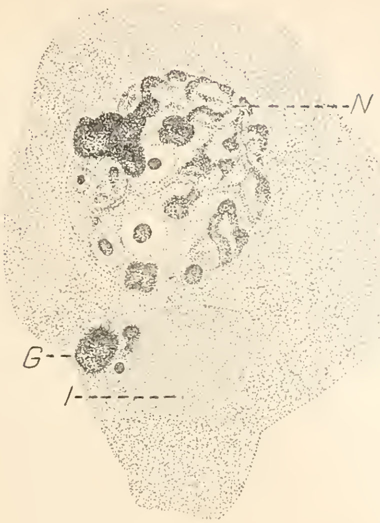
Plate I.

Fig. 1. Spermatid soon after the last maturation division. *G*, Golgi bodies; *N*, nucleus; *I*, idiosome.

Fig. 2. Spermatid showing increased size. *G*, Golgi body.

Fig. 3. Spermatid with the chromatin in the form of a spireme. *I*, idiosome; *G*, Golgi body.

Figs. 4, 5, and 6. Spermatid showing an abortive attempt to form chromosomes. *I*, idiosome; *G*, Golgi body.



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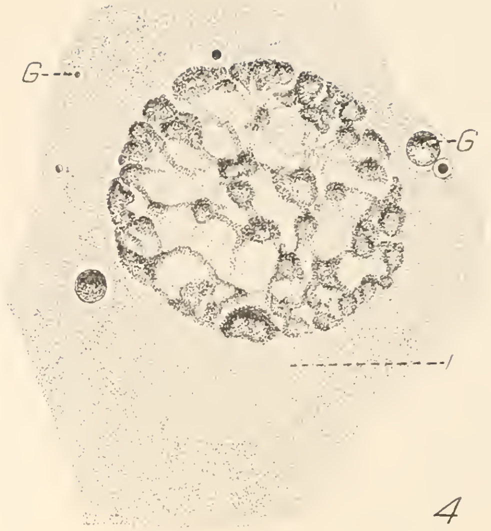

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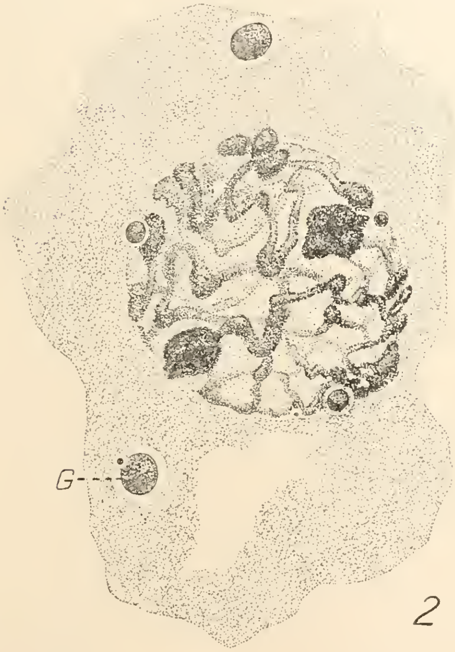

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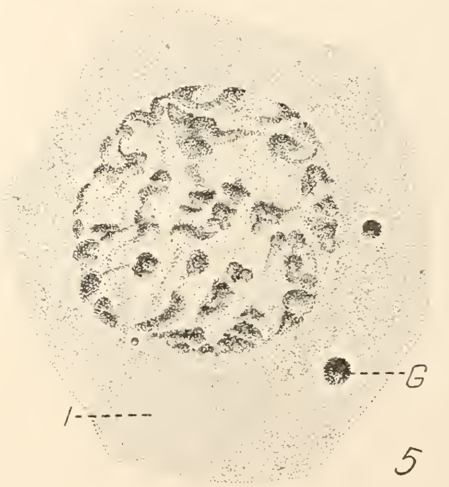

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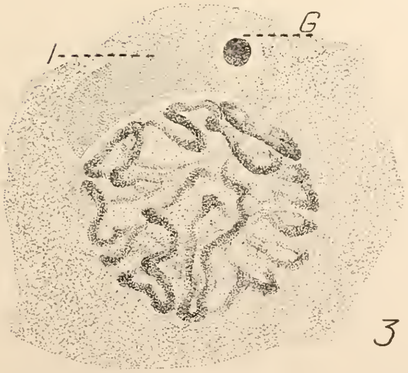

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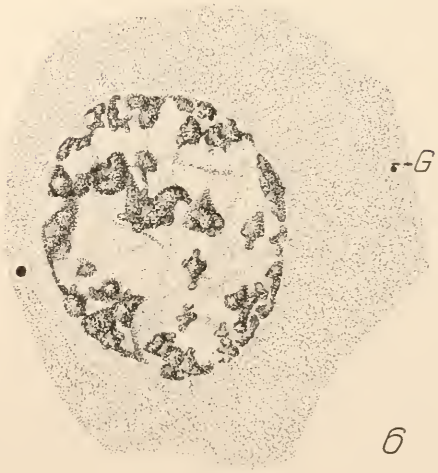

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MARY T. HARMAN AND FRANK P. ROOT.

Plate II.

Fig. 7. Spermatid showing the chromatin finely granular and the beginning of the contracting of the entire cell.

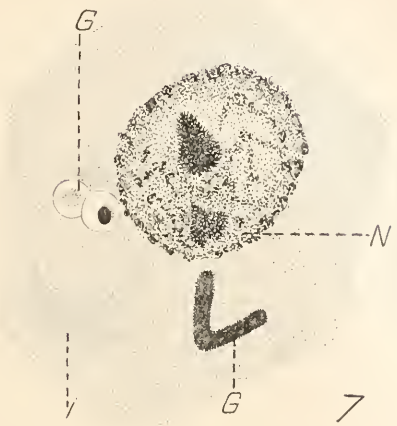
Fig. 8. Spermatid showing the idiosome closely applied to the nucleus and the appearance of the idiosphere. *I*, idiosome; *NE*, idiosphere; *G*, Golgi body.

Figs. 9 and 10. Spermatids showing a great reduction in size. *I*, idiosome; *NE*, idiosphere.

Figs. 11 and 12. Spermatid showing the ovoid shape which is the beginning of the elongation. *I*, idiosome; *NE*, idiosphere; *G*, Golgi body; *C*, cytoplasm.

Figs. 13 and 14. Spermatid showing the beginning of the elongation of the idiosome and the idiosphere. *I*, idiosome; *NE*, idiosphere; *N*, nucleus; *C*, cytoplasm; *G*, Golgi body; *A*, acroblast.

Figs. 15, 16 and 17. Spermatids showing the elongation of the nucleus and the spreading out of the cytoplasm in a fan-shape. *A*, acroblast, *N*, nucleus; *C*, cytoplasm.



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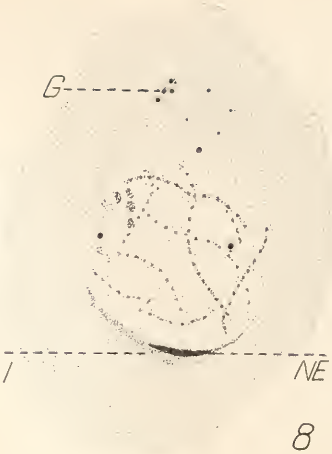

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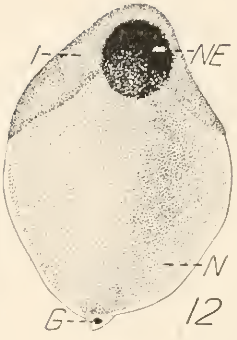

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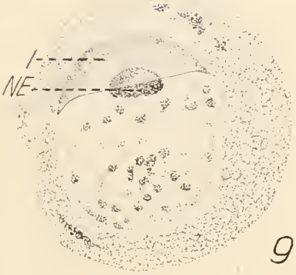

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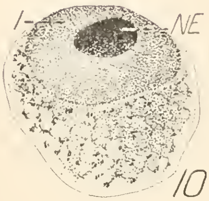

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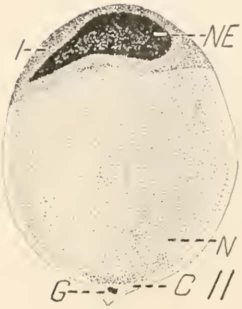

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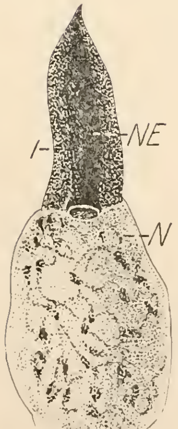

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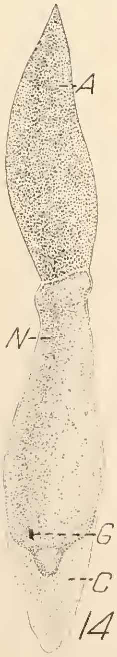

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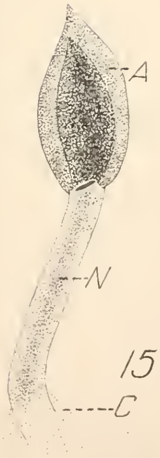

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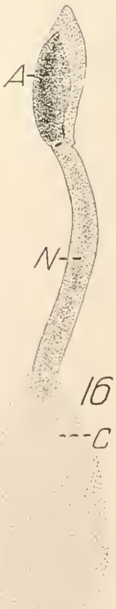

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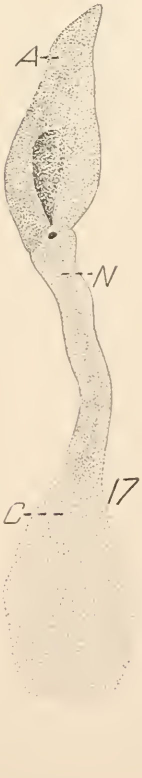

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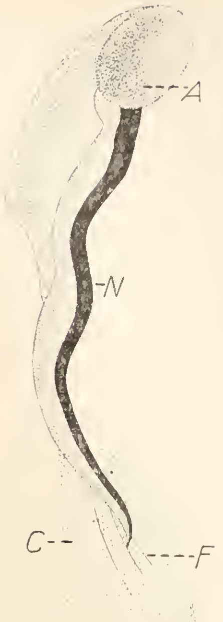
PLATE III.

FIGS. 18 AND 19. Spermatids showing appearance of filaments from the nucleus and the extension of the acroblast to the side of the elongated nucleus. *A*, acroblast; *N*, nucleus; *C*, cytoplasm; *F*, filaments; *G*, Golgi body; *Gr*, granules.

FIG. 20. Sertoli cell with some of the associated spermatids in an elongated form. *S*, spermatids; *N*, nucleus; *AC*, spermatogonial cell.

FIGS. 21 AND 22. Spermatids, a continuation of the development shown in Figs. 18 and 19. *A*, acroblast; *N*, nucleus; *F*, filaments; *C*, cytoplasm; *Gr.*, granules.



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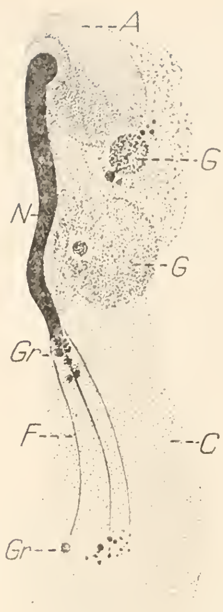
A
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F
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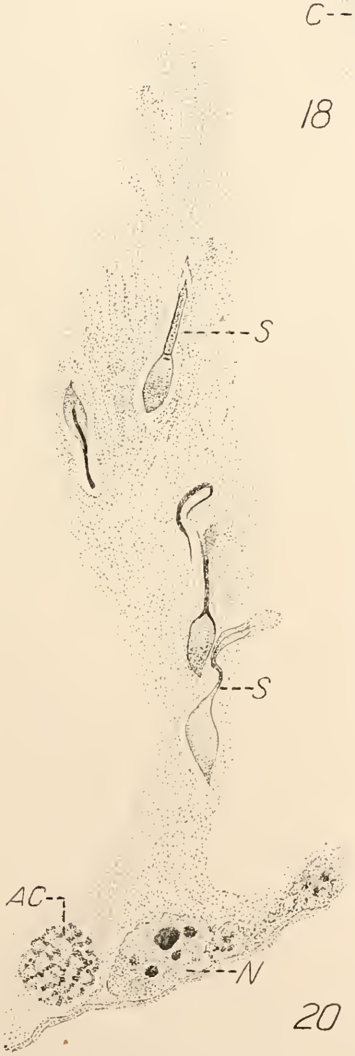

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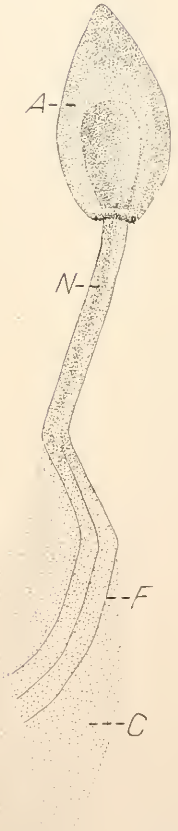

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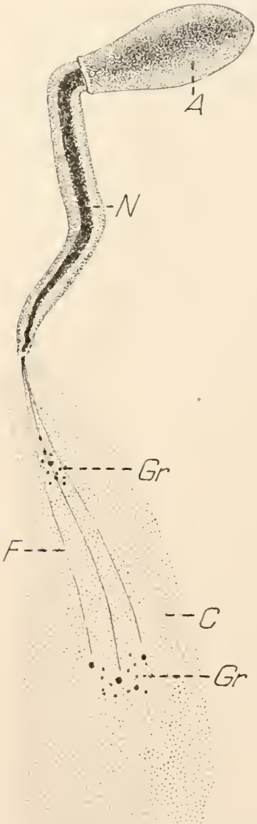

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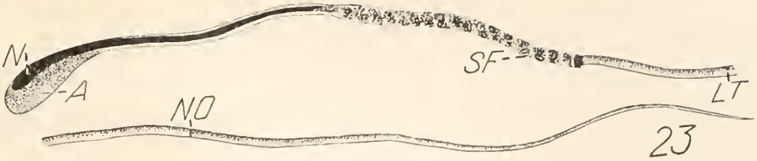
PLATE IV.

FIG. 23. Spermatid almost transformed, viewed from one edge. *N,* nucleus; *SF,* spiral filament in formation; *A,* acrosome; *LT,* tail segment; *NO,* node.

FIGS. 24, 25, AND 26. Mature spermatozoa. Fig. 24 viewed from convex surface, Fig. 25 from side angle and Fig. 26 from edge of head. *A,* acrosome; *N,* nucleus; *R,* residual cytoplasm; *SF,* spiral filament; *T,* tail; *NO,* node.



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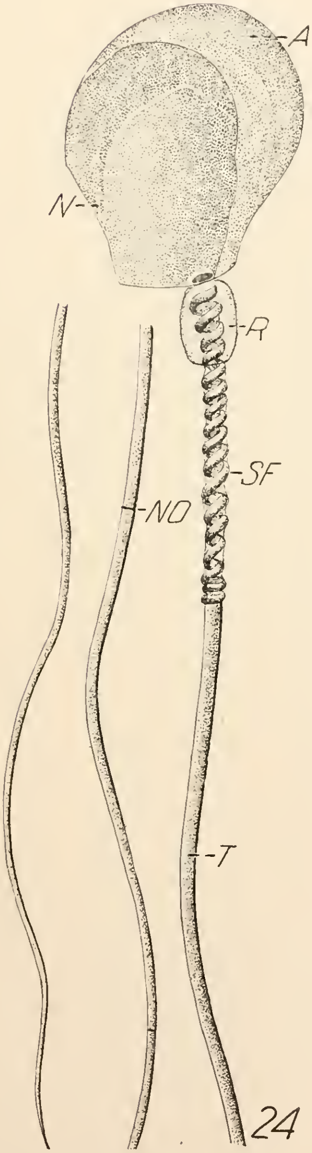

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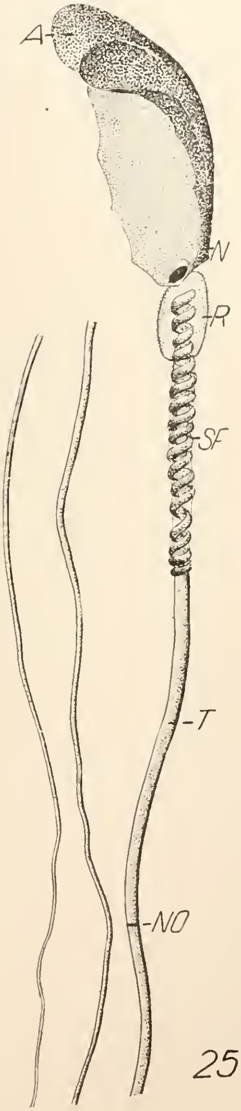

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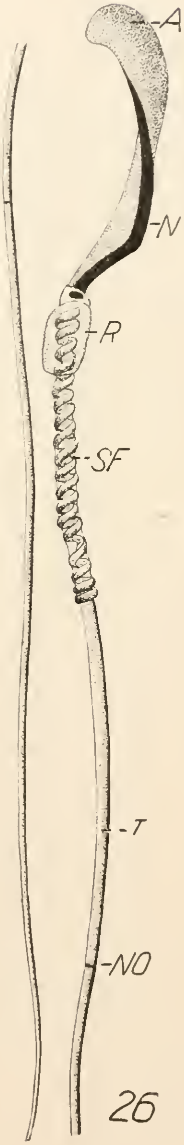

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STUDIES ON THE SECONDARY SEX CHARACTERS OF CRAYFISHES. VIII. MODIFIED THIRD ABDOMINAL APPENDAGES IN MALES OF *CAMBARUS VIRILIS*.

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The first and second abdominal appendages of males are habitually modified in *Cambarus* for the purpose of copulation. The individual parts of the first pair of appendages are fused and twisted and lie compactly in a groove on the ventral side of the thorax. The appendages of the second abdominal segment resemble the typical swimmeret in general plan (Figs. 1 and 6). However, the protopodite is elongated and heavier, the basal unsegmented portion of the endopodite is likewise reinforced, bearing a conspicuous triangular shoulder. The terminal segmented portion of the endopodite is much reduced. The remaining swimmerets are unusually quite typical.

There is apparently only one published record of a modified third abdominal appendage. Moenkhaus, Proceedings of the Indiana Academy of Science, 1903, pp. 111 and 112, describes a specimen of *Cambarus virilis* bearing such a modification as follows: "The first and second pairs of appendages were modified in the usual way and in no way differed from corresponding appendages in the normal male of the same species. The additionally modified third pair resemble in plan almost exactly the second pair. The exopod and the segmented flabellum of the endopod are much less reduced and much more extensively provided with feathered setae than the second pair. They are about the same size and in position converge and fit against the second pair of appendages much in the same manner that these do against the first. Whether they were in any way functional I am, of course, unable to say." Another specimen with a modification similar to but not so fully developed as the one described by Moenkhaus was collected by Dr. H. J. Van Cleave of the University of Illinois and appears in his collection.

Since crayfishes are in such common use as laboratory subjects it seems likely that any considerable occurrence of this aberrancy would have been noted and described. The writer has examined thousands of crayfishes during the past seven years, always with the object of finding peculiarities in the secondary sex characters and while large numbers of specimens have been found in which other aberrancies occurred, not one was found with this type of peculiarity until the lot described came to light.

A collection of several hundred specimens which had been taken from the Fox River between Green Bay and DePere, Wisconsin, during the summer of 1927, was being used in the Zoölogy Laboratory at Northwestern University. A specimen having peculiar appendages was discovered by chance and the writer then examined the entire lot. Forty-six of a total of three hundred and forty-two males were found which had third abdominal appendages modified somewhat like those of the second abdominal appendages. No other peculiarities were noted among the males, but one female in seventy possessed a pair of copulatory hooks on the third walking legs like those of the male. The latter type of aberrancy is the most common and it is surprising to find a type that is apparently rare in greatly superior numbers.

Description of Specimens.

The male specimens with the modified third abdominal appendages are about thirteen and a half per cent. of the total number examined. They range in length from 79 to 107 mm. Twenty-eight are second form and eighteen are first form males. A fairly complete series is represented in the aberrant appendages. In some, the third abdominal appendages varies only in the presence of a slight projection upon the inner border of the endopodite between the basal unsegmented and the terminal segmented portions (Figs. 2 and 3), while at the other end of the series the modifications are practically like those of the second abdominal appendages (Figs. 4 and 5). There is apparently no relation between the extent of modifications of the appendages and the size of the animals. In form I. specimens the angles upon the shoulder of the aberrant appendages are sharper and stronger than those of form II., but this might have been expected since the same is true of the usual modified appendages in normal form I. and form II. males.

The first and second abdominal appendages are normal in every respect in all the specimens.

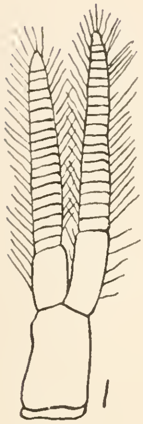
Discussion.

In attempting to find an explanation for the large occurrence of a rare aberrancy, age, accidental embryonic development, effect of environment or peculiar genetic constitution might be suggested at first thought as causal factors. The fact already noted in this description that size, and therefore age, and degree of development of the peculiarities in the appendages are independent would seem to eliminate age as a factor. Accident might be called upon to account for a specimen or two but scarcely for so large a number as is represented here. It has yet to be shown that environment has played any part in the development of the secondary sex characters of crayfishes, nor indeed, in modifying them.

Peculiar genetic constitution seems to be the logical factor here. It has already been shown for other aberrant conditions in sex characters of crayfishes that there is a strong tendency for the development of one type of peculiarity in one locality and the present case is another instance of the same tendency. It has been argued in these other instances that the peculiarity might easily arise and perpetuate itself as a mutation and the explanation is again offered for the case in hand.

It does not seem likely that this modification has any functional significance. Specimens more radically modified in other sex characters have been functioning normally and there is no reason to believe that this slight peculiarity would make males any more efficient nor that it would interfere with copulation.

The series offered in the specimens here, from the slightly modified to the most completely modified may give a clue as to the evolutionary changes through which the normal second abdominal appendages came in the course of their development. This is speculative, of course, but we have here an actual series ranging from a practically unmodified third abdominal appendage to one which almost exactly duplicates the normal second. Unless the highly peculiar second abdominal appendages arose with all their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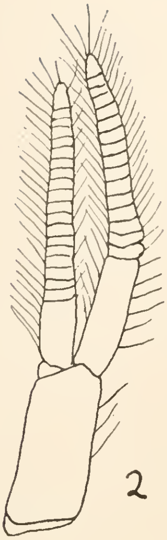
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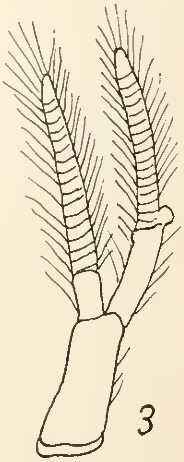
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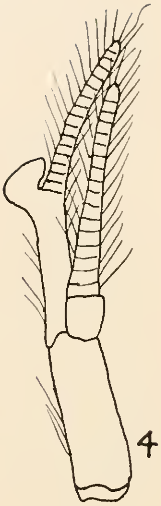
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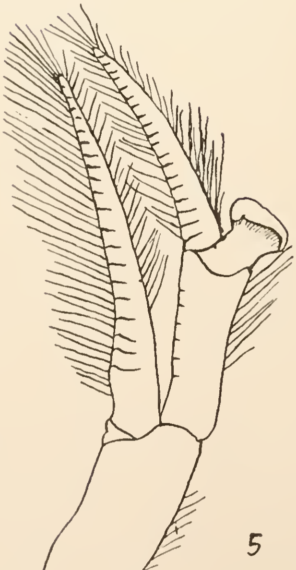
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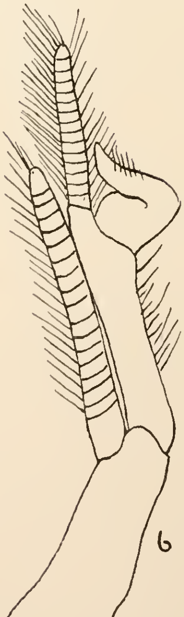
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peculiarities fully formed in one stage it is easy to believe that they arose through a series of changes such as is represented here. The first stage would be represented by the development of a low projection on the inner surface of the endopodite between the unsegmented basal portion and the segmented terminal portion. Subsequent changes would involve an enlargement of this spur and a molding of it until it had assumed the shape found in the normal second appendage of the male. Other changes would involve an elongation and an enlargement of the propodite, and an enlargement and a strengthening of the basal portion of the endopodite together with a reduction of the terminal segmented portion of the endopodite.

In aberrant females having first abdominal appendages modified like those of males the second abdominal appendages are also sometimes modified. Such aberrant females are rare but even in a small number various degrees of modification are shown in the second appendages. These second abdominal appendages are identical in their structural peculiarities with the third abdominal appendages described here and are similar also in that they show various stages of development.

EXPLANATION OF FIGURES.

Note: All figures are drawn to the same scale.

FIG. 1. Unmodified left third abdominal appendage of normal male.

FIG. 2. Left third abdominal appendage of aberrant male measuring 88 mm. Callosity on endopodite is showing first stage of development.

FIG. 3. Left third abdominal appendage of aberrant male measuring 82 mm. Callosity on endopodite much larger.

FIG. 4. Right third abdominal appendage of aberrant male measuring 80 mm. The protopodite is longer, the basal portion of the endopodite elongated and the shoulder upon the endopodite is more prominent.

FIG. 5. Left third abdominal appendage of aberrant male 103 mm. in length. Modifications almost equal to those of the normal second abdominal appendage.

FIG. 6. Left second abdominal appendage of normal male measuring 98 mm.

NATURAL HISTORY OF SHIPWORM, *TEREDO NAVALIS,* AT WOODS HOLE, MASSACHUSETTS.

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WABASH COLLEGE.

From the Marine Biological Laboratory, Woods Hole, Mass.

SECTION I. OCCURRENCE.

The common species of shipworm at Woods Hole, as identified by Kofoid and Clapp, is *Teredo navalis.* The date of its first appearance in this region is not known. Verrill lists it in his "Invertebrate Animals of Vineyard Sound and Adjacent Waters" (1871). Whatever its history in American waters may have been, it is now known to occur throughout the entire North American coast from Alaska to Labrador.[1] The present study has been carried on during the past four years and in that time no other species has been collected. It is known, however, that *Bankia fimbriata* occurs in this region, although in comparatively small numbers. During the year this work was first undertaken it was difficult to obtain *Teredo* in sufficient numbers for satisfactory study, but this is not an indication that the species is not abundant in New England waters. The reason for an apparent scarcity is that shipworms are inaccessible, being, for the most part imbedded in piles and permanent structures. Subsequently, by putting out suitable timbers during one summer to be studied the next, it has been an easy matter to obtain *Teredo* in abundance. Lobster pots[2] and 2 × 4 stakes have been found to be the most convenient. If these timbers are exposed to the water during the latter part of the summer they are found to contain sexually mature worms by the beginning of the breeding season the following June. The 2 × 4 stakes give best results if exposed during July or early August, but the smaller timbers are liable to complete destruction before winter if put out early in the summer.

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[1] Nelson, '22, speaks of an infestation of *Teredo navalis* in Barnagat Bay, New Jersey, as a sudden outbreak. He is probably in error in thinking that this species arrived so recently on the New England coast.

[2] Lobster pots are constructed of small slats about the size of ordinary plasterer's lath, 2 in. broad and ½ in. in thickness.

Teredo do not grow large in small timbers such as are used in the construction of lobster pots, but are easily removed from such small strips of wood, thereby facilitating study. The size attained depends upon the degree of crowding. To ascertain the size to which *Teredo* will grow, it is necessary to supply larger pieces of wood and 2 × 4 stakes are excellent for the purpose. With a drawing knife it is possible to expose the entire burrow in a few minutes because *Teredo* tunnels with the grain of the wood, usually within half an inch of the surface. A study of such stakes has shown that *Teredo* larvæ attack the wood in great numbers at the mud line but less and less abundantly from the bottom to the surface of the water. Three fourths of the *Teredo* burrows in an exposed timber occur within two or three feet of the mud line. Very few are found more than four feet above the bottom.

SECTION II. ANATOMY, PHYSIOLOGY AND BEHAVIOR.

The anatomy of *Teredo* has been accurately described by several early investigators and more recently the shell and digestive tract have received attention by Miller and Lazier, whose admirable work is published in four papers. It is sufficient here to say that the shipworm has the structure of an ordinary lamellibranch in which the body is much elongated and in which the bivalve shell is highly modified in adaptation to the burrowing habit. In one particular my observations are not in agreement with those of Miller. He attributes the formation of the rings of growth, the rasping ridges, and denticles of the shell to alteration or fluctuation in the food supply which, according to his conception, results in corresponding periods of slow and rapid growth. This may account for the annual rings of growth of certain mollusks and has been so interpreted, but it could hardly account for the rings and ridges on the shell of this young animal which adds two rings per week in the early stages of its development. These sculpturings of the shell which adapt it to burrowing are undoubtedly due to the action of little tongues of mantle tissue which are pushed up over the edge of the shell during deposition of the shell material. This process of shell sculpturing was observed in the large lamellibranch *Atrina rigida* (Grave, '09). The peculiar form and pattern of the shell is specific and is a matter of inheritance, but the building process is due to the peculiar manipulation of the mantle and not to alternate periods of starvation and plenty.

The physiology of digestion has been studied particularly in recent years by Dore, Miller and Potts.

Potts ('24) corroborates the work of Dore and Miller ('22) in showing that as the shipworm burrows through the wood it swallows the chips and derives some nourishment from them. A large section of the digestive tract seems to be devoted entirely to the digestion of wood (the cæcum and liver). Potts believes that wood is the only food of *Teredo* but Miller shows that the digestive tract contains diatoms as well as wood. The burrow mainly serves as a means of protection.

As the *Teredo* grows it enlarges its burrow proportionately until at maturity it may be 16 inches in length and have a diameter of 3/8 of an inch (40 × 1 cm.). A pile or other exposed piece of timber may be honeycombed with *Teredo* tunnels without showing on the surface that it is infested. The only opening of the burrow leading to the outside is the minute pore through which the young *Teredo* entered the wood as a metamorphosing veliger. Although less than .35 mm. in diameter and therefore too small to be seen readily by the unaided eye, it is through this passage that the siphons are protruded to obtain respiratory currents and food other than wood. The shipworm feeds upon minute organisms derived from water currents that pass over its gills for respiration, just as in ordinary lamellibranchs. It is in fact an elongated lamellibranch, whose burrowing shell covers only its anterior tip, leaving most of the body and the siphons unprotected except for the wooden shell-lined burrow.

Character of the Burrow.

The burrows are always lined by a calcareous substance, except at the anterior end, where further excavation is taking place.

This shell-like material is secreted by the general surface of the body or mantle. It has been suggested that this lining of the burow not only makes a smooth surface, but shuts out wood acids as well as external enemies which might otherwise injure the soft body of the animal. Even the outer pore-like opening is lined with this secretion and is divided transversely by a partition, so that the siphons protrude through two minute pores just large enough to transmit them. While the shipworm is not feeding, or when it is disturbed, the siphons are withdrawn and the external openings are plugged by two curious horny pallets, as they are called, situated one on each side of the siphonal region. See Figure I.

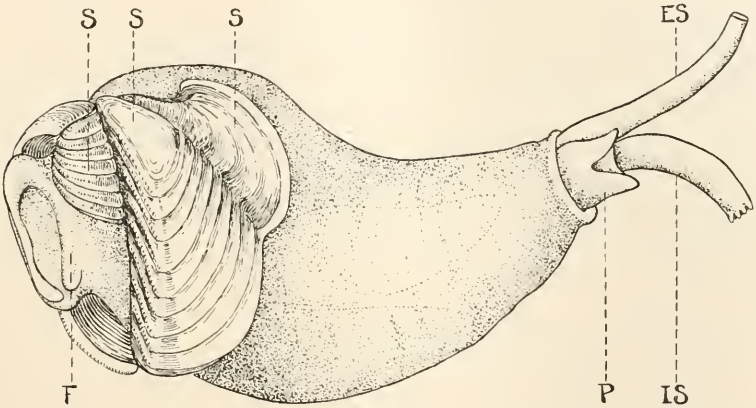

FIG. I. Young *Teredo,* length 2 cm., age five weeks from metamorphosis; drawn by camera lucida. S shell, F foot, i. s. incurrent siphon, e. s. excurrent siphon, p. pallet.

Effect of Adverse Conditions. (Repairing the burrow, etc.)

In case the tunnel is broken by accident, or by the wearing away of the surface of the wood from any cause, the adjacent glands secrete shell substance in greater abundance and mend the breach. The integrity of the burrow is carefully preserved. In case adverse conditions arise which make the environment difficult either from enemies or poisons in the water, or from overpopulation by its fellows, this shell substance is secreted in the form of a heavy casing, not only on the sides, but over the anterior burrowing end as well. This is the invariable reaction of

Teredo to adverse external conditions, the most common cause of which is the crowding of individuals in small timbers. As a consequence, the wood becomes extremely fragile, a mere shell, so porous that enemies, such as bacteria and parasitic protozoa, find entrance and menace the life of the community. Under these conditions the worms die within the first year. It may be, too, that wood is an essential part of their diet, but it is more probable that the trouble is a lack of adequate protection against adverse conditions and dangers from without.

No *Teredo* ever molests the burrow of another. When two come close together they face about and proceed in another direction, thus avoiding each other. When they become so closely crowded that further burrowing would infringe upon a neighbor, growth seems to stop. The size attained depends upon the amount of crowding. As stated above, the *Teredo* responds to these conditions by greatly thickening the shell lining of its burrow on the front as well as on the sides so that the whole is strongly encased. However, it is at best a brittle affair and parasitic protozoa and bacteria are admitted which soon destroy the occupant. The protozoan *Architophrya* (a holotrich) is always abundant in such situations.

It is difficult to see how growth may cease and the animal survive, but it is perfectly clear that *Teredo* three months old living in crowded situations are often less than one fifth as large as others of the same age growing under better conditions. The stunted worms, though packed closely together are frequently all alive and reproducing. As many as seven young *Teredo* per square inch have been observed in test blocks although the average is by no means so high. When these worms all become two or three inches long, a crowded group results unless they happened to have entered a large timber which permits of unlimited expansion.

Shipworms rarely go from one board to another, no matter how closely the boards are applied to each other. Only two exceptions to this rule have been observed among the thousands of burrows studied. They seem to avoid anything that threatens to interrupt the continuity of their tunnels.

Teredo seems not to orient to gravity since it burrows downward about as frequently as upward. The burrow of a single individual often shows that there is no tropistic response of this kind. If in tunneling downward a *Teredo* approaches the end of the timber, another *Teredo* burrow or a knot, it may turn directly about and proceed in the opposite direction, paralleling the first part of its burrow. By some means it is able to detect any nearby surface of the wood and avoid it. Two *Teredo* tunnels may approach within an eighth of an inch of each other, but they remain quite separate. They have some sense also which warns them, when approaching the end of a timber, to face about before reaching the end, retreating usually at a point 5 to 10 mm. from the tip.

Section III. The Breeding Season.

My interest in *Teredo* dates from 1922 when the National Research Council suggested the study of the breeding season of this species and appropriated funds to meet preliminary expenses. The results of this study were reported at the Washington meeting of the American Association for the Advancement of Science in 1924, and an abstract was printed at that time. The publication of the paper as a whole was deferred until the study of various details could be completed.

The fact that the female carries the young embryos in the gill chamber for a short time makes an accurate study of the breeding habits a comparatively easy matter. It may be ascertained at a glance whether a female is carrying embryos or not and the presence of eggs or embryos in the suprabranchial chamber is conclusive evidence of recent spawning. A further useful indicator is that of color. The eggs and young embryos are pure white, but they gradually take on a dark gray color with age.

The first spawning at Woods Hole occurs from the first to the middle of May, and the last about the middle of October. During 1925 eggs were first obtained on May 15 and these were in a late cleavage stage when discovered. Two of twenty females examined had spawned at this date. In 1926 eggs were first obtained on May 16. Two of the twelve females examined had spawned, and the embryos were in the gastrula stage of development. Frequent previous examinations in April and May had shown no spawning individuals.

During the fall of 1925 and 1926 special trips were made to Woods Hole in order to determine the extreme limits of the breeding season. At this time an effort was also made to learn how late in the fall veligers were metamorphosing and entering wood. On September 22, 1926, numerous females, both in Eel Pond and at the Cayadetta Wharf in Vineyard Sound, were carrying embryos in various stages of development. On October 10, of sixty *Teredos* taken from Eel Pond, none were carrying embryos, while five of twenty five taken from the Sound had quantities of veligers in their gills. The embryos of one of these were late trochophores or early veligers and repeated observation on the rate of development in *Teredo* has shown that these would normally be carried from ten days to two weeks longer. None were found carrying embryos on November 4. These and other data show that the breeding season in Eel Pond ended two weeks earlier than in Vineyard Sound. The difference in temperature is apparently the cause of this diversity in the duration of the breeding season, Eel Pond being approximately two degrees colder during the fall than the deeper water of the Sound. Kofoid noted a similar difference in the breeding season in various parts of San Francisco Bay where wide stretches of shallow water become several degrees warmer in early spring and cooler in the fall than the deeper portions of the same body of water. His estimate of two weeks difference is no doubt conservative. Observations just completed at this writing show that the first spawning by *Teredo* in Eel Pond in 1927 occurred on May 1 and in Vineyard Sound on May 12. Spawning occurred in each case when the water had reached a temperature of approximately 11° C. (between 11° and 12° C.). Since spawning ceased in Eel Pond on October 1 and in Vineyard Sound about October 15 we have the same variation due to temperature difference and the total spawning season for *Teredo* at Woods Hole is shown to be nearly or quite five months in duration.

It should be explained that the larva has a free swimming period of approximately two weeks after leaving the suprabranchial chamber of the mother before it is ready to enter wood. In accordance with the fact that veligers are carried by the mother as late as October 20 in Vineyard Sound, one would expect to find that wooden structures are being entered by the metamorphosing veligers until the first of November. The facts, however, do not bear out this expectation. The last date on which veligers successfully metamorphosed and attacked wood in Eel Pond was September 23, whereas larvæ were no doubt present until about October 5. Lobster pots placed in Vineyard Sound on October 10 were entered by metamorphosing veligers. It is certain that larvæ are present in the water in Vineyard Sound until November 1 or the last week in October. In other words, larvæ are present in the water at least two weeks after the last ones successfully attack wood. The reason for this is not evident. The cilia of the swimming mechanism of the larva possibly become less and less active as the water cools, with the result that mortality among the last generation of larvæ of the season is high. In *Bugula* also the last larvæ of the season fail to metamorphose, but not to so great an extent as is the case with *Teredo*.

An examination of the gills of a large number of *Teredo* on November 4 showed a spotting of these organs as if the last embryos contained had been resorbed. It is quite likely that the belated ones lose ability to swim and therefore remain inactive and disintegrate in the gill chamber. (This may not be the correct explanation of the cause of the failure of the last embryos of the season to metamorphose.) The larvæ of *Bugula* and those of certain hydroids continue to metamorphose successfully into November and the latter into December although dependent upon cilia for locomotion.

The data in hand indicate that the breeding season of *Teredo* at Woods Hole extends from about May 10 to October 10 or possibly to October 15, a period of five months.

Fecundity.

Teredo is tremendously prolific. Each female spawns three or four times in a season. The number of eggs produced varies with the size of the individual and is estimated to be from one to five millions. At the end of the season the female seems to be exhausted. Many molluscs survive for several years but *Teredo* dies during the second year as test blocks have shown repeatedly. This unusual fecundity may explain the early loss of vitality.

As evidence that the female *Teredo* spawns every four or five weeks, the following data are offered. Several cases of this kind were observed.

June 20, 1925. Two large females which were carrying gray veligers, were ready to spawn a second time. The ovaries were large and distended with eggs which were full size and fertilizable.

June 24, 1925. Two among several females examined had spawned a second time this season, numerous late veligers mixed with cleaving eggs were found in the suprabranchial chamber.

Periodicity.

One of the specific objects of this study was to ascertain the characteristics of the breeding season, whether or not there is a lunar or other periodicity in the production or shedding of the gametes. It was made apparent during the first year's study that no lunar periodicity occurs in the spawning of *Teredo*. From the beginning to the end of the breeding season, the water contains abundant larvæ in all stages of development. The records of examinations of hundreds of stakes and lobster pots indicate that larvæ are abundant in the water ready to attack any exposed timber each day of the summer. The evidence bearing on this point is derived from two types of experiments which are here described in some detail because other workers have stated that the spawning of *Teredo* is periodic and that definite broods mature at definite times.

1st. The following tables show that no periodicity in the spawning by this species occurs. Of a large number of ship worms that may be examined at any time during the summer, some will be found to carry cleaving eggs, some gastrulæ, and some trochophores, some young veligers and some typical veligers, thus showing that spawning is continuous and not synchronous.

TABLE I.

Teredo EXAMINATIONS 1925, JULY 1.

Material from Lobster Pot Placed in Water Aug. 16, 1924. Eel Pond.

Females carrying unspawned eggs	4
Females carrying cleaving eggs in gill chamber	1
Females carrying young veligers in gill chamber	1
Females carrying typical veligers in gill chamber	4
Mature males with active sperm	7
Total	17

TABLE 2.

Teredo EXAMINATIONS 1924, JULY 5.

Material from Lobster Pot Placed in Water Aug. 20, 1923. Cayadetta Dock.

Females carrying mature eggs	5
Females carrying immature eggs	1
Females carrying cleaving eggs in gill chamber	7
Females carrying gastrulæ in gill chamber	5
Females carrying young veligers in gill chamber	4
Females carrying typical veligers in gill chamber	5
Mature males having motile sperm	7
Immature males	3
Total	37

TABLE 3.

Teredo EXAMINATIONS 1924, JULY 19.

Material from Lobster Pot Placed in Water Aug. 20, 1923. Cayadetta Dock.

Females carrying eggs	8
Females carrying cleaving eggs in the gill chamber	2
Females carrying gastrulæ in the gill chamber	11
Females carrying young veligers in the gill chamber	3
Females carrying typical veligers in the gill chamber	5
Mature males with active sperm	5
Total	34

TABLE 4.

Teredo EXAMINATIONS 1924, AUG. 10.

Material from Lobster Pot Placed in Water Aug. 20, 1923. Eel Pond.

Females carrying eggs	1
Females carrying cleaving eggs in the gill chambers	1
Females carrying blastulæ or gastrulæ	4
Females carrying early veligers	2
Females carrying typical veligers	5
Females carrying a few veligers in the gill chambers, apparently spent	1
Mature males with abundant active sperm	12
Total	26

These four tables show that spawning takes place at all times during the month and not synchronously. They show conclusively that there is no lunar or other periodicity such as that sometimes caused by variations of temperature. Attention is called to the fact that the spawning of these animals took place not in the laboratory, but normally in their natural habitat.

It is also apparent from these tables that there are no "broods" or special times of infestation of exposed timber. As further evidence on this point the test blocks (lobster pots) were put out every ten days during the summer and all became infested with metamorphosing *Teredo* larvæ almost at once, certainly within a day or two after exposure, as numerous experiments on rate of growth show. At Woods Hole the first larvæ settle and begin to burrow toward the end of June (June 20). From that time on until early fall the water contains a copious supply of swimming larvæ ready to burrow into any exposed wooden structure.

T. C. Nelson in his report for the year 1923, Table 5, page 208, concludes on very meager and insufficient data that one brood of larvæ settled in Barnegat Bay in June and that a second brood matured some time between July 26 and September 4. The evidence derived from my experiments covering four years show that there are no broods but rather a continuous entrance of timbers by larvæ maturing throughout the breeding season. The evidence of many experiments shows that one can not depend upon green timber or even seasoned 2 × 4 stakes for such experiments, as they may remain uninfested for weeks for no apparent reason. Seasoned lobster pots, however, regularly became infested either the day they were exposed to the water or very soon thereafter. This is possibly due to the horizontal position of the timbers in the water, as contrasted with stakes standing vertically. The answer to the question whether *Teredo* larvæ enter wooden structures in broods at special times or continuously has important practical bearings as well as scientific interest.

It is also apparent from the data of these tables that Nelson's statement that there are five hundred females to one male, does not hold for the Woods Hole region. Females outnumber males but by no means to so great an extent.

Kofoid has shown that the number of larvæ in any particular region depends upon the extent to which infested timber is present. Regions far from wooden warves have relatively few larvæ in the water. I was able to show that *Teredo* is much more abundant at the Cayadetta Wharf than in Eel Pond, the ratio being approximately 2:1. The distance between these locations is less than one hundred yards and the difference in numbers in this case is not due to a difference in the amount of wood present. The biological conditions in the more or less isolated Eel Pond are clearly different from those of the open waters of Vineyard Sound because species inhabiting them are different to some extent, as shown in another paper (See *Bugula*).[1] A study of these conditions is contemplated but at the present no adequate explanation is suggested unless the large amounts of formalin and other poisons and oils from the supply station seriously affect the Eel Pond water at times. There are, however, differences in natural conditions. The tidal currents outside, at any rate, are much stronger than those in Eel Pond.

SECTION IV. EMBRYOLOGY, AND RATE OF DEVELOPMENT.

The extensive contributions of Sigerfoos and Hatschek give satisfactory descriptions of embryological development so that I shall avoid duplication and emphasize only facts that are new.

The egg of *Teredo* is comparatively small and white in color. It measures in extreme limits from .050 mm. to .061 mm. with an average diameter midway between these figures. The oviducts open into the suprabranchial chambers which are extensive and serve as brood pouches. When the eggs are extruded they are retained in the suprabranchial chambers for a period of two or three weeks, during which time they pass through the early stages of development. When liberated into the sea water they are typical lamellibranch veligers, vigorous and hardy. A large female may liberate from 500,000 to 1,000,000 eggs at a single spawning, so that the gill chambers are tightly packed with embryos distributed in two parallel rows along the sides of the slender elongated body. The approximate age of embryos can be estimated by their color since they gradually change from white to a dark muddy gray during development.

The embryo is not parasitic upon the mother, but the egg will not develop outside the gill chamber. Ripe eggs were several times removed from the gonads and artificially fertilized in an attempt to observe them in development. Development was initiated but no egg cleaved beyond the sixteen cell stage, and many stopped at the two, four, and eight cell stages. Development in these cases was extremely slow and cleavage was irregular and abnormal. Eggs fertilized at six P.M. had reached the eight cell stage at 9 P.M. It is probable that development in this species is normally slow, but this rate can hardly be considered normal. Very young embryos in the two and four cell stages were several times found in the suprabranchial chambers and these when removed developed no better than the artificially fertilized eggs. In common with artificially fertilized eggs, they finally became viscid and adhered to the containing dish. It was found also that blastulæ and gastrulæ would fare no better. They failed to develop into swimming larvæ. Late trochophores and early veligers on the other hand continued to develop normally when removed from the gill chamber to sea water. Veligers removed prematurely showed great vigor and swimming ability, and were several times kept for two weeks in sea water. Veligers withstand much rough treatment and survive in poorly aerated and even foul water. Some were kept in glass aquaria and fed on diatoms for three weeks, but to what extent they metamorphosed and entered the wood that was provided was not learned.

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[1] *Bugula flabelleta* lives readily in Eel Pond but will not thrive in the adjacent waters of Vineyard Sound, while the reverse is the case with *B. turrita*.

The gastrula is invaginate, similar to that of many other molluscs and annelids that produce small eggs with little yolk. The trochophore is especially interesting because in adaptation to its parasitic mode of life, it fails to develop a strong protoroch. The cells which normally develop this larval swimming organ are undoubtedly present and distributed in a broad equatorial band similar to that of many molluscs, and they are more extensive than in most annelids. The protroch is apparently present and was described by Hatscheck. I found it either absent or so feebly developed as to be easily overlooked. The trochophore is pear shaped or slightly elongated and on the average measures .059 × .060 mm. in length. As it begins to transform into the veliger, strong cilia develop on the velum, and the embryo becomes motile long before it is ready to be expelled into the sea water to shift for itself.

Duration of Larval Period.

Sigerfoos failed to find free swimming veligers in the water and both he and Nelson speak of the habits and duration of the larva as being unknown. The larval period from fertilization to metamorphosis has usually been estimated at about one month. It is evident, however, that it varies somewhat with temperature being shorter in tropical and sub-tropical regions than at Woods Hole.

I have frequently found *Teredo* veligers, in various stages of development, settling upon horizontally placed boards and Nelson has more recently taken them in "tow," as well as hovering about piles ready to settle permanently. In fact, he corroborates the observations of Harrington that the mature veligers of *Teredo* are attracted to wood and wood extracts. The duration of the free swimming period has never been accurately determined. To give attention to this phase of the life history publication of this paper has been delayed until its study could be completed and verified. The evidence now at hand indicates that the entire developmental period from egg to metamorphosing larva, is about five weeks. At least half of this time is required for development in the gill of the mother, leaving for the free-swimming period not to exceed two or three weeks. The evidence on which this conclusion is based is derived in various ways but is indirect. Since the method and conclusion may be questioned, the data are explained in considerable detail in the following pages.

In 1925 the first eggs were laid between May 12 and May 15, while the first young metamorphosed *Teredo* were found in test blocks on July 2 and July 5. These young, metamorphosed shipworms measured .35 mm. to .5 mm. Evidence collected from many experiments carried out during the past two years shows that young *Teredo* of this size have spent from 15 to 18 days in the wood, or rather, that they settled and began to metamorphose and burrow 15 to 18 days previously. If we subtract 15 days from July 2 or 18 days from July 5, June 17 is the approximate date when these *Teredo* ended their careers as free swimming larvæ. Other young *Teredo* collected on July 7 measured 1 mm. and these are known to be three weeks old, or that three weeks had elapsed since they settled upon wood. Subtracting twenty-one days from July 7 gives the date June 17 when metamorphosis began. The total larval period is therefore between four and five weeks. Other data collected during 1925 lead to the belief that the time is more nearly five weeks than four. This method, though indirect, is accurate, and was repeated many times at the opening of the breeding seasons of 1925 and 1926. The experiments made to determine the rate of the metamorphosis and rate of early growth were also repeated many times during the past two years and are also reliable. They show conclusively that young *Teredo* which measure one millimeter are approximately three weeks old. The spring of 1925 opened unusually warm, although the winter was severe, so that animals came out of winter hibernation a few days earlier than usual. The effect of this was shown most markedly in the rapid somatic growth of many animals, but it also affected to a slight extent the breeding seasons of most animals. The date of first settling of *Teredo* larvæ at Woods Hole is usually about June 20, and the first spawning about May 10. The variation in the spawning season from year to year does not usually exceed two or three days but it may vary more than a week. There is evidence that some animals begin to breed only when the water rises to a certain temperature. This, however, is by no means a universal rule.

Rate of Growth.

The veliger of *Teredo* has the typical form common to lamillibranch larvæ, but is not so thick or nearly spherical as sometimes described. Young veligers taken from the gills in an early stage of development measure on the average .065 x .080 mm. Five specimens taken from two individuals measured as follows: .060 x .080 mm.; .065 x .080 mm.; .065 x .082 mm.; .070 x .083 mm.; .070 x .085 mm. These measurements represent the range of variation in length and breadth. One of these seen in edge view measured .082 x .05 mm., and an older one .09 x .05 mm. Veligers ready to begin their free swimming life, after spending two or three weeks in the gill of the mother, measure somewhat larger, as the following examples show: .070 x .090 mm.; .072 x .090 mm.; .075 x .085 mm.; .075 x .088. These measure-

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ments not only indicate some growth but also that a considerable variation exists in the relative measurements. The range of variation in ratio of length to breadth and also in length of hinge line is great. Veligers fed upon diatoms for one week measured from .077 x .090 mm. to .081 x .093 mm. The size attained at the time of metamorphosis was not learned, but Nelson ('23) gives it as .25 mm. in length. I have collected several hundred young metamorphosing *Teredo,* which had burrowed into wood, varying in age from two to three weeks after settling. These range in size from .35 mm. to 1 mm. The smaller ones in two weeks have almost completed metamorphosis and have from two to three rasping ridges or rings of growth on the shell. Individuals three weeks old have four rings of growth and a typical *Teredo* shell. At three weeks of age the *Teredo* is practically spherical and its burrow, when exposed by cutting away the surface of the wood, is a hemispherical pit. The young worm now begins to elongate rapidly and at the end of one month its burrow measures from 5 to 7 mm. in length, and has a diameter of 2 to 2.5 mm. The shipworm when expanded fills its burrow so that, in measuring the rate of growth, the size of the burow may be taken as the correct measure of the enclosed worm. When the shipworm is removed from its burrow, it contracts to one half or two thirds of its expanded measure. Tables 5 and 6 show the rate of growth from the egg to adult size, and need not be described in detail. Measurements were taken every three or four days, and the rate of increase in size from day to day was found to be surprisingly rapid.

It should be noted that the ages given in the tables include only the time that elapsed from the time of settling. If the age from the egg is desired, about thirty-five days should be added to these figures to include the time from fertilization to the end of the free swimming period. The larval period is excluded in the following description and from the tables.

Growth during the first twenty-five days seems small but when the minute size of the animal at the beginning is taken into account, the growth is not slow. From one month to five months the increases shown during the intervals of three or four days, between measurements, are seen to be remarkably great. For

Table 5.

Rate of Growth of *Teredo navalis* (Summer).

Measurements of the Largest Burrows of Specified Ages.

Date and Period of Growth.	Age.	Size of Burrows in Length and Widest Diameter (Metric).	Approximate Length in Inches.
July 16 to July 26....	10 days	No visible *Teredo* burrows.	
July 16 to Aug. 3....	18 "	No visible *Teredo* burrows.	
July 26 to Aug. 13...	18 "	.35 to .5 mm. x .35 to .5 mm.	.014 to .02 in.
July 16 to Aug. 8....	23 "	.35 to .5 mm. x .35 to .5 mm.	.014 to .02 in.
July 16 to Aug. 11...	25 "	.5 to .1 mm. x .5 to 1 mm.	.02 to .04 in.
July 26 to Aug. 20...	25 "	1 to 1.5 mm. x 1 to 1.5 mm.	.04 to .06 in.
July 16 to Aug. 13...	28 "	2 to 3 mm. x 1 to 2 mm.	.08 to .12 in.
July 16 to Aug. 16...	30 "	5 to 7 mm. x 2 to 2.5 mm.	.2 to .28 in.
July 16 to Aug. 18...	33 "	8 to 10 mm. x 2 to 3 mm.	.32 to .4 in.
July 16 to Aug. 23...	38 "	14 to 17 mm. x 3 mm.	.56 to .7 in.
July 16 to Aug. 28...	43 "	35 to 45 mm. x 4 mm.	1.4 to 1.8 in.
July 16 to Sept. 1....	46 "	50 to 57 mm. x 4 to 4.5 mm.	2 to 2.3 in.
June 20 to Aug. 20...	60 "	70 to 75 mm. x 4 to 4.5 mm.	2.8 to 3 in.
July 3 to Sept. 6.....	65 "	80 to 90 mm. x 4 to 4.5 mm.	3.2 to 3.6 in.
June 22 to Sept. 3....	72 "	100 to 120 mm. x 4.4 to 4.8 mm.	4 to 4.8 in.
June 22 to Oct. 20...	130 "	140 to 170 mm. x 6.5 to 7 mm.	5.6 to 6.8 in.
June 22 to Dec. 1....	160 "	175 to 200 mm. x 7 to 7.5 mm.	7 to 8 in.
July 1923 to July 1924	One year	250 to 400 mm. x 7.8 to 9.4 mm.	10 to 16 in. x $\frac{5}{16}$ to $\frac{3}{8}$ in.

Note:—The left hand columns of Tables 5 and 6 represent the time of exposure of timber to the sea water. Thus, if we consider the first item of Table 5, timbers were exposed to sea water on July 16 and examined for *Teredo* on July 26, making ten days as the maximum age of the infesting *Teredo* as indicated in the second column of the table. Column three gives the measurements of the infesting *Teredo* if any.

The measurements given in these tables are actual cases and not averages. Many more were measured than are given here but the data given are considered typical.

In all cases the larval period is omitted. The age from fertilization may be approximated by adding thirty-five days to the age as given here.

Timbers exposed forty-three days, July 16 to August 28 contained sexually mature worms. Other similar data show that *Teredo* under favorable conditions becomes sexually mature in six weeks at Woods Hole. The item second from the bottom of Table 5 shows that *Teredo* does not reach adult size during the first season (June 23 to December 1). No growth takes place after December 1. (See also Table 6.) The last item of the table shows that adult size is attained in one year (July 1923 to July 1924). The largest specimen found at Woods Hole measured forty centimeters in length. It was precisely one year old.

example, the length of the largest burrows at twenty-five days is 1.5 mm., at thirty days 5 to 7 mm., at thirty-eight days 14 to 17 mm., at forty-three days 35 to 45 mm. Davenport claims that growth in size is partly due to swelling by the absorption of water. It is easier to account for this phenomenal growth in this way than to suppose that the change in size represents only protoplasmic growth and actual cell multiplication.

It has been repeatedly found that *Teredo navalis* at Woods Hole reaches sexual maturity and spawns from six to eight weeks after entering the wood as a metamorphosing larva. The youngest to spawn were six weeks old, and their burrow measured one and one half inches (38 mm.) in length. They spawned in abundance at the age of two months when they measured 2–2½ inches (50 to 63 mm.) in length. Sexual maturity is reached long before adult size is attained, since a fully developed shipworm measures from 12 to 16 inches (30 to 40 cm.) in length.

The larvæ that metamorphose first in a season almost reach adult size by December 1, the largest ones, measuring from seven to nine inches (17.5 to 22.5 cm.) in length. During December, January, February and early March ship worms are practically dormant and do not grow perceptibly. Then they may be said to awaken and by the first of July the oldest have reached maximum size. The largest specimen found at Woods Hole measured 16 inches in length and ⅜ inch in widest diameter (40 x 1 cm.). It grew in a 2 x 4 test take which was in the water from July 1, 1923 to July 1, 1924. Others measuring from 12–15 inches are common. Larvæ which enter the wood later in the summer, even to October 1, lie dormant over winter and resume growth the following spring. It was shown that growth is greatly retarded after the first of November, especially on the part of the youngest *Teredo*. Table 6 shows the rate of growth for the entire year including the winter. It has already been stated that *Teredo* reaches adult size in one year and dies during the second year.

Teredo navalis is said to grow to a slightly larger size in subtropical climates than at Woods Hole. As stated above the largest specimen found in this northern locality in four years' study measured forty centimeters in length and one centimeter in greatest diameter.

TABLE 6.

RATE OF GROWTH OF *Teredo navalis* (EFFECT OF WINTER UPON GROWTH).

Measurements of the Largest Burrows of Specified Ages.

Period of Growth or Time of Exposure of Timber.	Age.	Size of Burrows in Length and Widest Diameter (Metric).	Length and Width of Burrows in Inches.
Nov. 1 to Dec. 1.........	1 month	No teredo infestation.	
Oct. 15 to Dec. 1.........	$1\frac{1}{2}$ "	No teredo infestation.	
Oct. 1 to Dec. 1..........	2 "	No teredo visible.	
Sept. 15 to Dec. 1........	$2\frac{1}{2}$ "	No teredo visible.	
Sept. 1 to Dec. 1.........	3 "	No teredo visible.	
Aug. 20 to Dec. 1........	$3\frac{1}{2}$ "	35 to 45 mm. x 4 to 4.5 mm.	$1\frac{1}{4}$ to $1\frac{3}{4}$ x $\frac{5}{32}$ x $\frac{3}{16}$ in.
Aug. 10 to Dec. 1........	$3\frac{2}{3}$ "	75 to 81 mm. x 4.5 to 5 mm.	3 to $3\frac{1}{4}$ in. x $\frac{3}{16}$ to $\frac{7}{32}$ in.
Aug. 1 to Dec. 1.........	4 "	100 to 110 mm. x 6.2 to 7 mm.	4 to $4\frac{7}{16}$ in. x $\frac{1}{4}$ x $\frac{9}{32}$ in.
July 20 to Dec. 1.........	$4\frac{1}{3}$ "	112 to 144 mm. x 6.2 to 7 mm.	$4\frac{1}{2}$ to $5\frac{3}{4}$ in. x $\frac{1}{4}$ to $\frac{9}{32}$ in.
July 10 to Dec. 1.........	$4\frac{2}{3}$ "	150 to 210 mm. x 6.2 to 7 mm.	6 to $8\frac{1}{2}$ in. x $\frac{1}{4}$ to $\frac{9}{32}$ in.
July 1 to Dec. 1..........	5 "	175 to 250 mm. x 6.2 to 7 mm.	7 to 10 in. x $\frac{1}{4}$ to $\frac{9}{32}$ in.
July 1 to July 1..........	12 "	250 to 400 mm. x 7.8 to 9.5 mm.	10 to 16 in. x $\frac{5}{16}$ to $\frac{3}{8}$ in.
Sept. 12 to July 1........	Winter +	48 to 70 mm. x 3.3 to 4.1 mm.	$1\frac{9}{10}$ to $2\frac{4}{5}$ in. x $\frac{1}{8}$ to $\frac{1}{6}$ in.
Sept. 23 to July 1........	Winter +	12 to 31 mm. x 2.5 to 3.3 mm.	$\frac{1}{2}$ to $\frac{5}{8}$ in. x $\frac{1}{10}$ to $\frac{1}{8}$ in.

Note:—Table 6 shows, primarily, the amount of growth attained by *Teredo* during the first season up to the beginning of the hibernation period (Dec. 1). It shows not only that the earliest larvæ of the season fail to reach maturity (adult size) the first season, but also gives the amount of growth attained by the later larvæ of the season as well.

The first five items of this table show that *Teredo* larvæ which enter the wood after September 1 will not attain sufficient size to be detected in the wood up to December 1. Timbers exposed to the water as late as August 20 on the other hand are likely to contain *Teredo* measuring 35 to 45 mm. in length by the end of the growing season (Dec. 1).

The last two items of the table show that timbers exposed to the water between Sept. 12 and Sept. 23 become infested by *Teredo* and that they appear in the wood the following July, although they remain too small during the winter to be detected.

The latest infestation observed at Woods Hole occurring in Eel Pond was Sept. 23, and in Vineyard Sound Oct. 10. It may sometimes occur somewhat later than this, since the point was not sufficiently investigated. Table 6 shows that growth in the late fall is very slow compared with summer growth as given in Table 5 and data not tabulated show that practically no growth takes place in *Teredo* at Woods Hole between Dec-1 and March 1. Some observations indicate that the gonads begin to proliferate extensively before there is detectable body growth in the spring.

SUMMARY.

Teredo navalis occurs in abundance at Woods Hole and vicinity and has been known there for many years.

The breeding season extends from about May 10 to October 10. Spawning begins in the spring when the water reaches a temperature between 11° and 12° C. Spawning by each female occurs several times during the season. No lunar periodicity in spawning occurs and there are no broods caused by synchronous spawning.

The eggs are retained in the gills of the mother during cleavage and early larval development.

The time required for the fertilized egg to complete larval development to metamorphosis is approximately five weeks at Woods Hole. About half of this time is passed in the brood pouch and half as a free swimming veliger.

When eggs and early embryos are removed from the gills they do not develop normally.

The trochophore of *Teredo* is non-motile, having either a feebly developed protroch or none.

Teredo navalis reaches sexual maturity in six weeks or two months after metamorphosis when it measures four or five centimeters in length. It reaches adult size in on year, and dies during the second year. The largest specimen collected in four years measured forty centimeters in length and one centimeter in greatest diameter.

The rate of growth during the summer months and also during the winter was determined and tabulated. Certain habits of shipworms were also observed and recorded.

APPENDIX.

Practical Measures.

Because of numerous inquiries by lobstermen and owners of small boats concerning methods of preventing damage by shipworms, a series of experiments was made on the effect of drying upon *Teredo*. These experiments were not extensive but sufficient to show that simple but effective precautions may be taken.

Teredo larvæ first begin to enter wood between June 20 and June 25 and stop about October 10. Shipworms do not enter wood at any other time during the year. Little or no damage is done to wood until it has been in the water one full month. The largest of the young shipworms are only one fourth of an inch long at the end of one month but they attain a length of one inch in six weeks. It is, therefore, advisable to dry lobster-pots and boats once per month and leave them out of the water exposed to the sun for one week. This is especially true in July and August when most of the damage is done. Shipworms in small timbers are killed by five days' exposure to sunlight, but 2 x 4 stakes and larger timbers require from a week to ten days for drying sufficiently to kill all of the worms.

EXPERIMENTS.

(1) Infested lobster-pot lath:
  (*a*) After exposure in air to bright sunlight for 1 day, seemed to be dry but some of the infesting shipworms were still alive.
  (*b*) After exposure in air to bright sunlight for 2 days, some shipworms alive.

  - (*c*) After exposure in bright sunlight for 3 days, all shipworms dead.
- (2) Infested lobster-pot lath:
  - (*a*) After 7 days on shelf in laboratory, all worms contracted and shrunken, some of which regained plumpness and normal activity when placed in sea water. Spermatozoa and larvæ taken from these shrunken worms showed activity.
  - (*b*) After 10 days on shelf in laboratory—all worms, sperm and larvæ dead.
- (3) Infested 2 x 4 stakes:
  - (*a*) After exposure in air to sunlight for 5 days; many shipworms dead, some living.
  - (*b*) After exposure in air to sunlight for 7 days; all shipworms dead.
- (4) Infested 2 x 4 stakes:
  - (*a*) After exposure in air in shade for 7 days; many worms dead but some living.
  - (*b*) After exposure in air in shade for 10 days; none living.

Note:—If infested 2 x 4 stakes are exposed in air in the shade but kept wet some worms may live for several weeks.

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STUDIES OF HUMAN TWINS.

I. Methods of Diagnosing Monozygotic and Dizygotic Twins.

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Introduction.

This is the first of a series of studies of human twins based upon a considerable collection of pairs taken from the environs of Chicago. These studies have been carried on in collaboration with Professors F. N. Freeman, K. J. Holzinger, and Mrs. Blythe Mitchell. The original objective of this research project was to secure an adequate collection of monozygotic and same-sexed dizygotic twins about whose diagnosis we could be certain. With this objective attained, it was proposed to make an intensive comparative psychological study of the two types of twins to determine, if possible, the influence of heredity and environment upon the various mental traits. This is an old and somewhat hackneyed problem, but one that has never been at all satisfactorily solved. It seemed to us, however, that all previous studies had been inadequate because methods of diagnosing the two types of twins were unsatisfactory. The one crying need then was for a satisfactory method of diagnosing monozygotic twins, and the working out of such a method was assigned to the present writer.

Collection and Classification of Material.

The objective set by the collaborators in this study was the collection of fifty pairs of identical twins and fifty pairs of fraternal twins. In order to simplify our task, we decided to eliminate the disturbing factor of sex dimorphism, and therefore confined our study to twins of the same sex, pairs in which the twins were both boys or both girls.

At first no selection was practised among same-sexed twins, but all cases were taken as they came. As each case was completed an informal vote of the three or four workers present was taken as to the category (identical or fraternal) to which the pair belonged. Rarely, if ever, was there any difference of opinion, but in about one tenth of the cases there was some uncertainty and these cases had to be studied more intensively.

It soon appeared that the collection of identicals and fraternals was not going evenly, the fraternals being more numerous. If our preliminary judgments as to their classification were accurate we would need to stop the collection of fraternals and collect only identicals during the last stages of the period of study.

When the adjudged "fraternals" mounted to fifty-two cases (consisting of twenty-four male pairs and twenty-eight female pairs) there were only forty-three "identicals" (consisting of twenty-five male pairs and eighteen female pairs). The sex ratio at that time was very close to normal expectancy: forty-nine male pairs to forty-six female pairs. The question arose as to whether the proportion of identicals to fraternals was running according to theoretical expectancy.

Various methods have been used to determine the proportion of monozygotic to dizygotic twins. One method involved the examination of the fetal membranes of considerable numbers of twin births in institutions where competent observers were able to secure these important diagnostic data. Spät in 1860 reported that, in a total of one hundred eighty-four cases of twins examined as to the membranes, 24.6 per cent. were monozygotic. Brem in 1891 reported 22.7 per cent. of monozygotic twins out of one hundred twenty-six twin births. Krahn in 1891 reports 19 per cent. of monozygotic twins among one hundred twenty-seven twin births, but includes as monozygotic two opposite-sexed pairs. Tigges found in 1896, 21 per cent. of monozygotic twins among fifty-two twin births, and Quenzel in 1894 reported 20.4 per cent. of monozygotics among one hundred eighty-one pairs of twins. These percentages range from 19 per cent. to 24.6 per cent.

A second method used by several investigators for computing the proportion of identical twins is statistical in character. The best known of these methods is Weinberg's "differential method."

In 1902 Weinberg described his method as follows: "Assuming that sex is determined at the time of fertilization and that about half of all zygotes will produce males and the other half females, it follows that there will be equal numbers of same-sexed as opposite-sexed fraternal twins. If, therefore, we double the number of opposite-sexed twins and subtract the product from the total of all twins, the remainder will represent the number of monozygotic twins."

Applying this method to large masses of twin data he found that the percentage of monozygotic twins varies from 23.4 per cent. to 31 per cent., the percentage differing in different countries. This agrees rather closely with the percentages determined on the basis of fetal membranes.

Recently Knibbs (1926) has worked out a formula for computing the number of monozygotic twins in the twin population, using data taken from the census of Germany. His formula is as follows: The ratio of monozygotic twins to all twins is $(M + F - P) \div (M + F + P)$, where M is the number of ♂ ♂ pairs, F the number of ♀ ♀ pairs, and P the number of ♂ ♀ pairs. This method gives 24.4 per cent. of monozygotic twins in Germany from 1906 to 1911.

Applying Knibbs' method to the extensive twin data for the United States that is presented by Nichols (234,497 ♂ ♂; 264,098 ♂ ♀; 219,312 ♀ ♀), we discover that 26.42 per cent. of this large group are monozygotic and that nearly 42 per cent. of all same-sexed twins are monozygotic.

The question now arises as to whether our small random collection of ninety-five pairs of same-sexed twins was composed of the expected number of identical and fraternal pairs. According to our diagnosis there were forty-three pairs of identicals and fifty-two pairs of fraternals—*i.e.*, 45 per cent. identicals instead of the expected 42 per cent. This is but a small discrepancy and may have two meanings: Either the random selection of twins has brought in two or three too many pairs of identicals or else some two or three of the pairs diagnosed as "identicals" should be classed as "fraternals." It is probable that the former explanation is correct, for it is very unlikely that the ideal ratio as determined on the basis of 717,907 pairs of twins would be realized exactly in the first ninety-five cases selected at random. In fact, the close approach to theoretical expectation actually realized is almost too close. The conclusion may then be drawn from this that our methods of diagnosing identical and fraternal pairs cannot be far astray.

In order to complete the proposed collection of fifty pairs of identicals and fifty pairs of fraternals, it was then necessary to select seven cases of certain identical twins. Two cases of fraternals were eliminated from the fifty-two cases of fraternals in order to get down to fifty cases. The two cases eliminated were chosen for the following reasons: In one case one twin had lost three fingers and his palm was so scarred that no adequate palm print could be taken; in the other case one of the twins showed up with an infected hand and no palm print could be obtained. Since, in our diagnosis of monozygosity, the palm prints were used as highly important criteria, it seems well to eliminate these two pairs in which the palm print evidence was incomplete. The two pairs eliminated were unequivocal cases of unlike fraternal twins.

We have now complete data on one hundred pairs of same-sexed twins, fifty of which have been classed as identicals and fifty as fraternals. No doubt some of our readers are wondering how we can speak so confidently about our ability to classify all of our cases as either identicals or fraternals. It may be said that the method was slow in taking shape and was arrived at only after intensive study of the materials.

Diagnosis of Monozygotic Twins.

The majority of workers on human twins seem to have despaired of arriving at an adequate classification of twins into clean-cut categories: monozygotic and dizygotic. Years ago Thorndike found so much difficulty with his cases that he came to the conclusion that all twins belong to a single series and have a similar origin. Lauterbach, 1925, after the study of nearly two hundred pairs of twins, found himself unable to separate the same-sexed pairs with any assurance. He tentatively classified 59 per cent. of the same-sexed twins as monozygotic, a percentage much too high, suggesting that he has included a good many cases of similar fraternal twins in his "identical" group.

The most recent study of twins is that of A. H. Wingfield (1928) who studied one hundred two pairs of twins selected at random from the public schools of Toronto and Hamilton, Ontario. Taking all pairs of twins as they came there were accumulated seventy-six like-sexed pairs and twenty-six unlike-sexed pairs. The expectation would be about 65 per cent. of like-sexed twins instead of about 74 per cent., the number found in this collection. It seems probable, therefore, that some unlike-sexed twins were overlooked. Wingfield made an attempt to separate the seventy-six like-sexed pairs into two groups, identicals and fraternals. His method was somewhat precarious. He classed as "identical" all those which seemed to himself and the teacher to have a higher degree of physical identity than siblings are likely to exhibit. "Only those pairs of twins showing practically indistinguishable physical traits, as judged by the teachers in the school and myself, were included in the identical group. While it is not absolutely certain that all pairs included in the identical group had identical heredity, the chances in favor of this being the case are very great." The fact that he classed as identical over 44 per cent. of all the twins in his group is surprising in view of the fact that the statistical expectation is only about 26 per cent. It seems probable then that Wingfield has included among the "identicals" several cases of similar fraternal twins. This is further suggested by the fact that he found a coefficient of correlation of only about + 0.90 for this group as compared with + 0.95 obtained for our identicals.

That it is possible to develop a method of distinguishing between identical and fraternal twins is strongly suggested by the fact that two European twin specialists claim to be able to make such a distinction with a high degree of infallibility.

Dahlberg (1926), in his monograph on "Twin Births and Twins from a Hereditary Point of View," makes this statement: "The following demands should be satisfied for a diagnosis of monozygotism for a grown-up pair of twins:

"1. That the appearance of the twins give an impression of very great resemblance or identity.

" 2. That during childhood, neighbors, school-fellows, etc., have had difficulties in distinguishing them and have sometimes confused them.

" 3. That the configuration of the ears does not show great dissimilarity.

" 4. That the finger prints show a certain high degree of similarity.

" 5. That the anthropological measurements do not show too considerable differences."

Siemen's method (1927) is somewhat more detailed and exacting. He takes the very sensible view that no single criterion of monozygotic origin is reliable, but that judgment in doubtful cases should be based upon identity in as many traits as possible. He emphasizes the rarity of really questionable cases. Many years of experience in the study of twins has developed in him such a degree of confidence in his method of diagnosis that he considers that he has been able to reach " a certain diagnosis in virtually every case of twinning."

He finds, as others have found before and since, that the great majority of all twins are either so completely alike or so markedly different that there is no question about their diagnosis. A careful study of the certain cases should furnish criteria for diagnosing the few doubtful cases. Thus a study of over a hundred pairs of unquestionable identical twins has resulted in the following " scheme " for diagnosing monozygosity:

A. Traits in which one-egg twins practically always agree and in which two-egg twins agree only very rarely:
  1. Hair color and form.
  2. Eye color.
  3. Skin color.
  4. Downy hair of the body.

B Traits in which one-egg twins differ only within narrow limits and in which two-egg twins usually differ more widely.
  5. Freckles.
  6. Appearance of blood in the skin.
  7. Follicular processes.
  8. Tongue (furrowed or not) and teeth.

C. Traits in which one-egg twins usually, and two-egg twins rarely show strong resemblance:

9. Form of face.
10. Form of ears.
11. Form of hands.
12. Body build.
13. Mentality.
14. Illness and abnormality.
15. Traits studied by special methods—finger prints, etc.

Our own method of diagnosis has been considerably influenced by the methods of Dahlberg and of Siemens, especially by the latter, but is somewhat different from any previously used. Our effort has been to combine the best features of all known methods.

After our own method was developed and while reading Wingfield's monograph, the writer noted a reference to a short note in *Science* by Taku Komai (1927) entitled "A Criterion for Distinguishing Identical Twins from Fraternal Twins." The criterion described has to do with finger prints and palm and sole prints of twins. "Generally speaking," he says, "the same hands or feet of the identical twins resemble each other more closely in their patterns than the two hands or feet of the same individual." This I have found to be very frequently true, but the formula needs modification, as will be shown below.

Our Own Method of Diagnosis.

The method of identifying monozygotic twins used in the present work may now be described in detail. A great deal of attention has been given to this matter, for we realize that the soundness of our conclusions as to heredity and environment depend upon the correctness of this diagnosis.

At the beginning, it may be said that in over 90 per cent. of our cases there was at no time any doubt as to their classification. The great majority of one type of twins are so strikingly similar that their monozygotic origin is obvious. Their resemblance is not confined to gross physical correspondence, but extends to tones of voice, gestures, and peculiar mannerisms. One soon becomes sensitized to the intangible correspondences of identical twins and diagnoses them almost at a glance. The great majority of the other type of twins strike one at once as entirely unlike, often being more different than average brothers or sisters. About these there is no question after the first glance. Our ability to diagnose cases improved during the course of our study and we found that there was no difficulty at all in diagnosing the last half of the pairs that presented themselves. Two of the very early pairs were diagnosed doubtfully that, when reëxamined after a year of experience, offered no difficulty at all. Two other cases were left uncertain because we allowed ourselves to be influenced by statements of the mother. About these cases there should never have been any question had the mother not been loquacious.

Out of one hundred two pairs of twins there was justifiable doubt about only six cases. These cases have all been diagnosed satisfactorily with the possible exception of No. 61, which still remains slightly uncertain.

The following are our criteria for diagnosing identical (monozygotic) twins.

1. They must be strikingly similar in general appearance including various intangible resemblances.

2. They must be essentially identical in hair color, texture and form.

3. They must have the same shade of eye color and form of iris.

4. They must have the same skin color and texture (complexion) except when one is more tanned than the other.

5. They must have no marked differences in features; shape of ears; shape, size and arrangement of teeth.

6. They must have hands of the same type and nearly equal in size.

7. The general microscopic character of the papillary ridges in fingers and palms must be essentially the same.

8. There must be stronger cross resemblance than internal resemblance in one or more of the details of finger and palm patterns.

9. The presence of reversed asymmetry in handedness or hair whorl in one twin is confirmatory evidence of monozygosity, but

its occasional presence in unlike twins is not to be taken as an indication of monozygosity.

A great deal of stress has been laid upon the diagnostic value of the palm and finger patterns. While this criterion alone is inadequate for certain diagnosis, it is surprising how few mistakes were made in our effort to diagnose monozygosity on this basis alone. In the first forty-two cases in which a judgment was attempted on the basis of palm and finger prints alone, there was disagreement in only two cases with the judgment based on general resemblance. Our method has been to classify all cases on the basis of the first six criteria and then to check this classification by criteria 7 and 8.

Palm and Finger Prints as Criteria.

The intensive study of palm and finger patterns is perhaps the best single diagnostic aid. After a scrutiny of the first thirty or forty sets of palm prints the writer began to notice an important fact about the palm and finger patterns of strikingly identical twins: namely, that, instead of showing mirror-imaging of patterns (involving the resemblance of the right hand of one to the left hand of the other) the two hands of one of the twins were direct duplicates in major features of the two hands of the other. Specifically, the right hand of one twin is more like the right hand of the other than like own left hand, and the left hand of one twin is more like left hand of other than like own right hand. Thus cross resemblance between the two twin individuals is stronger than resemblance between the two hands of the same individual.

Among twins that are somewhat less alike the same rule holds in a somewhat modified form. Thus right hand of one twin may be like right of the other, or left of one like left of the other, but the close resemblance does not extend to both sides. In still other pairs of twins in which one is distinctly left-handed, there is a reversal of asymmetry, so that the right hand of each twin is like the left hand of the other. *In every pair of obviously monozygotic twins the rule holds that there is stronger cross resemblance between the hands of one twin and those of the other than between the two hands of the same individual.* The same is true of ears, teeth, and other structures that show more or less asymmetry, but there is more detail in palm and finger prints and a more objective method of comparing them. In the case of the fingers the types of patterns have been formulated in all cases in order to obtain a qualitative basis of comparison, and the friction ridges in all patterns (following the method of Bonnavie, somewhat modified) were counted under binocular so that a quantitative comparison between the fingers of one hand and those of another is possible. In both qualitative and quantitative respects the rule that cross resemblance is stronger than internal resemblance holds, for identical twins.

The studies of palm main line formulæ and of the occurrence and varied expression of the six fundamental primitive patterns have been greatly facilitated by the study of a paper now in manuscript, the work of a considerable group of experts, entitled "A Study of Error in the Interpretation and Formulation of Palmar Dermatoglyphies," by Cummings, Keith, Midlo, Montgomery, H. H. Wilder and I. W. Wilder. Professor Cummings, evidently the guiding spirit of the group in this collaborative inquiry, has very kindly furnished me with a copy of the manuscript and has thus made it possible for me to study the palms of our twins with far greater efficiency than would have been possible without this assistance.

With few exceptions the same rules of cross resemblance apply to the palmar main lines and patterns that apply to finger prints. Most frequently the cross resemblance runs similarly in all four respects: in qualitative characters of finger patterns, in quantitative values of finger patterns, in palmar main line formulæ, and in the occurrence of palmar patterns. Sometimes the cross resemblance is obvious in only three of four respects, sometimes in two, or only one; but if it is greater between one hand of one twin and either the same or opposite hand of the other twin than in own hands, the rule is considered to hold good.

While it is of importance that the detailed analysis of the finger and palm characters of this collection of twins should be published, this is hardly the appropriate place for it. One or two separate papers devoted to a special presentation and analysis of these data are planned for subsequent publication.

At this time we must ask the indulgent reader to accept tentatively our criteria for diagnosing twins. With the publication of the complete data used in this diagnosis the methods used may be put to any test that seems necessary.

Applying the criteria of diagnosis above described to the six pairs of twins about which there was some doubt, three of them fell readily into the category of identicals and three were classified as similar fraternals. At the present time the writer feels quite confident as to the correctness of diagnosis of the whole collection. The cases that might be questioned by some are the three cases of similar fraternals just referred to. Before discussing the problems arising out of a study of identical twins, it seems advisable to devote a few paragraphs to the fraternal twins, especially to the three cases most difficult to diagnose.

The Diagnosis of Fraternal Twins.

Of the fifty-two pairs of fraternal twins in our collection, three may be classed as " similar fraternals," and twenty as " slightly similar fraternals," and twenty-nine as " unlike fraternals." None of the pairs show as much resemblance as the least similar of the identical twins. The only cases that could possibly be at all in question as to their classification are the three " similar " pairs, numbered 61, 15, and 74. Let us carefully scrutinize these rather crucial cases as to the possibility that they might be monozygotic twins of the less nearly identical sort.

Pair 61.—These girls at first impressed us with their similarity. They were dressed exactly alike, arranged their hair alike and had very similar coloring. In height there was but three eights of an inch difference; there were two and three fourths pounds difference in weight. Head length of A was 13.95 mm., of B 14.35 mm.; head width of A was 17.7 mm., that of B was 17.9 mm. The hair of both was in general rather similar, but that of B was a shade darker, softer, finer and not so heavy. Eye color was the same in both, a type of hazel. There was no difference in skin color. Ears of A were higher and narrower than those of B, and had a shorter lower lobe. A has fuller lips; B has the longer, more prominent chin. A holds eyes wide open; B has them nearly half closed. Bridge of A's nose more bowed than that of B. The teeth of the two differ rather sharply, the upper arch of B being narrower and the teeth crowded and irregular, while those of A are regular.

The finger print formulæ are decidedly different:

Left Hands.	*Right Hands.*
1, 2, 3, 4, 5	1, 2, 3, 4, 5
A—U, R, A, W, W	A—U, U, U, U, U
B—W, U, R, U, U	B—W, R, U, U, U

The quantitative values of the finger prints are:

A—right hand 24	A—left hand 28
B—right hand 38	B—left hand 25

All four palm main line formulæ are different and the patterns are also different.

Left Hands.	*Right Hands.*
A—(9.8.5″.5′) B.O.O.O.O.	A—(11.9.7 .5.′) B.O.O.L.O.
B—(9.8.5″.3) A.O.O.O.O.	B—(9.7.5″.3) C.O.O.O.O.

Both are equally right-handed and both have clockwise hair-whorl.

In spite of a superficial rather close resemblance, then, there is no indication that these twins have had a monozygotic origin. This was the most difficult case to diagnose, but there seems now no doubt that these twins are dizygotic in origin.

Pair 65.—This case was somewhat puzzling because the two girls are both rather peculiar in appearance and are similar in many peculiarities.

In height A is 57½ inches, B 56⅞ inches. In weight, A is 113¼ pounds, B is 111¼ pounds. Head length of A is 14.5 mm.; that of B is 14.4 mm.; head width of A is 17.7 mm.; that of B is 17.1 mm. Hair of both is the same in color and texture; eye color of both is of the same shade of blue; B has a lower brow and a sullen expression about the eyes, while A has a contented expression. The skin is somewhat more florid in B. The ears of the two differ greatly, B having much longer lower lobe. The hands differ in shape, those of A being broader and thicker. B has shorter, more turned-up nose, a distinctly wider mouth, fuller lips and fatter face. The teeth differ radically, the upper arch of B being wider and straighter across the front and with wider teeth.

The finger print formulæ read as follows:

	Left Hands.					*Right Hands.*				
	1	2	3	4	5	1	2	3	4	5
A	W	R	W	W	U	W	W	W	W	U
B	W	W	U	W	U	W	W	W	W	U

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The quantitative values of the finger prints are:

A—right hand	53		A—left hand	54
B—right hand	52		B—left hand	60

The palm formulæ are as follows:

	Left Hands.		*Right Hands.*	
A	(11.7.7. 3)	A.O.O.O.D.	(11.9.7 5′)	A.O.M.O.D.
B	(9.8.5″.5′)	A/B.O.O.O.D.	(11.8.7.5′)	O.O.M. L.O.

In several respects there is a little more resemblance between right palm and fingers of the two than to their respective lefts, but this does not extend to details. On the whole these two girls make an entirely different impression. One has a rather pleasing, happy expression, the other a sullen, lowering expression. The fact that B is ambidextrous in both finger and wrist tapping suggests that she might be the left-hand component of a monozygotic twin pair, but there are too many differences between them to permit such a diagnosis.

Case 74.—These girls have many traits in common, but show also some extreme differences. A's height is 59 inches; B's is 53¾ inches. A's weight was 70½ pounds; B's 66½ pounds. A's head width is 14.1 mm.; B's 13.5 mm. A's head length is 17.6 mm.; B's is 17.1 m.m Hair color, texture and crown whorl same in both. Eye color of both a gray brown, but A's eyes are distinctly grayer and B's browner. B's ears are distinctly larger and wider although her head is considerably smaller. A's eyes are wider spaced than B's. A's nose is larger, longer and different in shape. B's teeth are crowded and overlap in front, while A's are straight.

Finger print formulæ:

	Left Hands.						*Right Hands.*				
	1	2	3	4	5						
A	W	R	R	U	U	:	W	R	U	U	U
B	U	R	U	U	U	:	U	A	A	U	U

Quantitative values of finger patterns:

A—right hand	44		A—left hand	30
B—right hand	27		B—left hand	26

Palm formulæ:

Left Hands		*Right Hands.*
A—(11.7.7.3) O.O.O.O.D.	:	A—(11.9.7.3) O.O.O.L.D.
B—(11.7.7.3) O.O.O.O.O.	:	B—(11.7.7.3) A.O.O.O.O.

Here again the palm formulæ suggests a closer resemblance than actually exists, in that we have the same pattern for the two left hands, but the two palms of B also have the same pattern and are far more similar in detail. Nowhere is there stronger cross resemblance than internal resemblance. On the whole there can be no doubt that these are fraternal twins.

Apart from these three cases there are no decidedly similar twins among the fifty-two pairs in our collection. Twenty pairs are designated as "slightly similar" fraternal twins and the remaining twenty-nine cases are designated as "unlike" fraternal twins. The slightly similar fraternals show merely the degree of resemblance common among siblings, while the unlike fraternals seem to show hardly as much resemblance as do average siblings. Even the three cases of decidedly similar fraternals, except for their identity in age, are no more alike than are occasional siblings. On the whole then, there seems to be nothing about these fifty-two cases out of accord with their classification as fraternal, or dizygotic, twins. Hence there is now no ground for doubting the validity of our classification of the one hundred two pairs of twins used in this study, into the two categories, monozygotic and dizygotic.

SUMMARY.

1. The original objective of these studies was the study of the rôles of heredity and environment in determining mental capacities of various sorts.

2. The first essential was to learn how to diagnose with certainty the two types of twins, monozygotic and dizygotic.

3. Only about 25 per cent. of all twins are monozygotic. Collections that depart widely from this figure have probably been incorrectly diagnosed.

4. Only about 42 per cent. of same-sexed twins are monozygotic.

5. The method of diagnosis used in this study combines the best

features of the methods of Dahlberg, Siemens, and Komai. Certain refinements of technique are added, the details of which are explained in the text.

6. Out of a collection of one hundred two pairs of same-sexed twins, only six pairs caused any difficulty, three of which are now classified as monozygotic and three as dizygotic.

7. The details concerning the three "similar fraternals" are presented and the reasons for their diagnosis as dizygotic twins are given.

8. The result is that we have now a collection of fifty pairs of monozygotic and fifty-two pairs of dizygotic same-sexed twins accurately diagnosed. These are to be used for further biological and psychological study.

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STUDIES OF HUMAN TWINS.

II. Asymmetry Reversal, of Mirror Imaging in Identical Twins.

H. H. NEWMAN,

Introduction.

One of the most striking phenomena observed among monozygotic twins is that of the reversal of asymmetry between the individuals of a pair. Among armadillo quadruplets the present writer (Newman, 1916) found numerous instances in which a band or scute doubling occurred on the left side of one twin and on the right side of the other. Such cases call to mind the fact that in human double monsters (Siamese twins) *situs inversus viscerum* occurs in many cases. The same type of asymmetry reversal was noted by Swett and by Morrill in double-headed fish embryos. In separate identical twins in man it has been noted that the incidence of left-handedness in one twin of a pair is very much greater than among fraternal twins or in the general population of single individuals. Asymmetry reversal in the direction of crown whorl of the head hair seems to have about the same incidence in monozygotic twins, dizygotic twins, and single individuals as has left-handedness. These two expressions of asymmetry have been studied intensively in the present investigation and their significance will be discussed in some detail later.

Handedness an Expression of Asymmetry.

As an introduction to this study it seems well to examine the phenomena of handedness as it is found among human beings. In the first place, there are two distinct kinds of handedness: that which is genetically determined and that which is the result of twinning and therefore epigenetically determined.

Genetic handedness is evidently transmitted in such a way that any given zygote will give rise, when no twinning occurs, to a right-handed or left-handed single individual. There seems, however, to be varying degrees of right- or left-handedness. The majority of individuals, apparently about eighty per cent.

of single individuals, are definitely righ-handed; about four per cent. definitely left-handed, and the remaining sixteen per cent. partially left-handed or ambidextrous. The incidence of right- and left-handedness is about what one would expect if right-handedness is a dominant Mendelian unit character and left-handedness recessive. The ambidextrous individuals and those showing lesser degrees of left-handedness may be heterozygous individuals in which the dominance of right-handedness is incomplete.

The other type of left-handedness, quite different in origin and heritability, is that which results epigenetically as the result of the twinning. Such left-handedness, being a somatic modification would not be hereditary: it would be merely an expression of asymmetry reversal due to the development of a whole individual from a half embryo which had already become more or less differentiated in a left-handed direction before the separation into twins has taken place.

Thus in genetic right-handed embryos which undergo twinning after some asymmetry has been established, the left-hand half embryo would be the superior one and would give rise to a right-handed individual, since right-handed superiority is due to left-sided superiority in the brain. Conversely, in a genetic left-handed embryo, the right side would be superior and the left side the inferior side, in which case the left-handed individual would retain the genetic asymmetry and the right-handed individual would exhibit asymmetry reversal.

In embryos genetically ambidextrous the right and left sides would be equal and would produce twins both of whom would be ambidextrous.

Previous Data on Handedness in Twins.

A good deal of attention has been paid by various authors to the peculiar incidence of left-handedness in twins. Siemens (1924) found in thirty-seven pairs of identical twins twenty-six cases both right-handed, ten cases in which one was right-handed and the other left-handed, and one case where both were left-handed. In a later paper the same writer reported on a larger number of identical twins (the total number not given) in which there were twenty-one cases where one was right- and the other left-handed and three cases where both were left-handed.

Weitz (1924) found among eighteen pairs of identical twins, seven pairs composed of a right- and a left-hander, ten pairs both right-handed, and one pair both left-handed.

Dahlberg (1926) reports for sixty-nine pairs of identicals fifty-three pairs both right-handed, twelve pairs one left-handed, and four pairs both left-handed. Adding the three sets of cases together, we have one hundred and twenty-four cases of identical twins divided as follows:

89 pairs, both right-handed,	71.8 per cent.
29 pairs, one left-handed,	23.4 per cent.
6 pairs, both left-handed,	4.8 per cent.

Dahlberg has also studied the incidence of left-handedness in one hundred and twenty-eight pairs of dizygotic twins. The following figures indicate his results:

111 pairs, both right-handed,	86.7 per cent.
16 pairs, one left-handed	12.5 per cent.
1 pair, both left-handed,	0.8 per cent.

It will be seen that the incidence of left-handedness among identical twins is over twice as great as among fraternal, or four times as great in proportion to the number of zygotes involved, for a pair of identical twins involves only one zygote. Even among fraternal twins, the incidence of left-handedness is relatively high as compared with the general population, which is reported by Jones (1918) to be about four per cent. Jones' estimate, however, is probably much too low and takes account of only the most complete cases of left-handedness.

Lauterbach (1925) reports among fifty-seven same-sexed twins (not distinguished as to monozygotic or dizygotic origin) twenty pairs in which one was left-handed, about 35 per cent. of all cases. This is a higher incidence of left-handedness than any previously reported, especially when it is taken into consideration that the group examined consists of both identical and fraternal twins.

The most recent data on handedness in twins is furnished by Verschuer (1927). He found one or more left-handed individuals in 26.8 per cent. of seventy-nine pairs of identical twins and in 26.3 per cent. of the thirty-eight pairs of fraternal twins. They were distributed as follows:

Identical Twins.	*Fraternal Twins.*
58 both right-handed.	28 both right-handed.
15 one right- the other left-handed.	10 one right- the other left-handed.
5 both left-handed.	0 both left-handed.
1 one right-handed, the other ambidextrous.	

The percentage is rather low as compared with those of others, particularly those of Lauterbach and the present writer, but the difference is probably due to the inclusion of only the cases of complete left-handedness. The percentage of pairs showing left-handedness among fraternal twins is exceptionally high and not in accord with the findings of others. Possibly the relatively small number of cases may be the cause of this discrepancy. Even more probable, it seems to me, is the inclusion among fraternal twins of a few of the least similar identical twins among whom left-handedness is common.

Criteria of Handedness.

It is by no means a simple matter to diagnose left-handedness. There are many cases, of course, where the twins are (or were at an earlier period) obvious left-handers, but there are also many cases where congenital left-handedness is obscured by training the right hand and suppressing the left. Such cases often result in a sort of ambidextrality in ordinary manipulations. In our work we have used as a test of handedness speed in tapping with wrist and fingers. In all cases of complete left-handedness the tapping tests confirm the left-handed diagnosis. It appears to be safe then to use the tapping tests to reveal native left-handedness obscured by right-hand training or various degrees of partial left-handedness.

A good many cases of partial left-handedness were revealed by tapping tests. Among identical twins, in addition to the eleven pairs showing complete left-handedness, there were thirteen pairs (both of whom considered themselves right-handers) in which some degree of left-handed superiority was revealed in one or both members of the pair. In three of these pairs both members

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were shown to be partially left-handed, and in two pairs both members were definitely ambidextrous.

Among fraternal twins, in addition to six pairs in which one individual was completely left-handed, there were five pairs in which one individual was partially left-handed, two pairs in which both were partially left-handed, and two pairs in which one individual was right-handed and the other ambidextrous.

Assuming that all these cases represent grades of left-handedness we have added to the seventeen pairs showing complete left-handedness twenty-two pairs showing partial left-handedness, a total of thirty-nine out of one hundred pairs in which some degree of left-handedness appears in one or both members of a pair. This high percentage would be much like that found by Lauterbach (35 per cent.) if we omitted the cases of ambidextrality.

Crown Whorl an Expression of Asymmetry.

As is well known, the head hair at the crown twists or whorls in either a clockwise or a counter-clockwise direction. The great majority of individuals show clockwise hair-whorl, and therefore clockwise asymmetry may be considered as the normal and counter-clockwise asymmetry as the reversed asymmetry. Various writers have called attention to sporadic instances of reversed crown whorl, and a few cases have been described for identical twins.

Only one writer, however, has thus far made a systematic study of crown whorl in twins. Lauterbach (1925) in a study of resemblances and differences in twins has presented some very interesting data. Out of fifty-seven pairs of same-sexed twins there occurred fifteen pairs in which one or both twins showed counter-clockwise hair-whorl. In one of these cases both twins were counter-clockwise. This means that about twenty-six per cent. of the pairs of same-sexed twins showed asymmetry reversal in hair-whorl. In addition to these, there were six cases showing double crown in which one half of the whorl has a clockwise and the other a counter-clockwise direction. These cases are possibly comparable to ambidextrality in handedness and should probably be listed as a form of asymmetry reversal. Adding these six cases, the percentage of pairs showing more or less reversed hair-whorl among same-sexed twins comes to nearly

37 per cent., not unlike the percentage of left-handedness in the same set of twins, which was 35 per cent.

In our own collection of one hundred pairs of same-sexed twins there are in all ninety-five pairs in which it was possible to determine the hair-whorl. In five pairs (three identicals and two fraternals) the kinky or closely matted character of the hair rendered diagnosis of hair-whorl extremely difficult or impossible. Among the identicals there were twenty pairs showing some form of asymmetry reversal in crown-whorl. In fifteen pairs one twin showed clockwise and the other counter-clockwise whorl, in three pairs both twins were counter-clockwise, and in one pair one twin had a double crown and the other a clockwise whorl. The remaining twenty-seven diagnosed pairs showed clockwise hair whorl in both twins.

Among fraternal twins there were but five pairs having any form of asymmetry reversal in hair-whorl. In four of these pairs, one twin was counter-clockwise, and in one pair one twin had a double crown and the other a clockwise whorl.

As in the case of handedness, there are doubtless instances of incomplete asymmetry reversal that are not recognizable. Probably some of the crowns diagnosed as slightly clockwise or indefinite may be cases of partial asymmetry reversal.

Crown-whorl has one advantage over handedness as a criterion of reversal of asymmetry in that it is not subject to modification by training and is therefore a somewhat surer sign of asymmetry reversal than is left-handedness.

The Relation between Handedness and Crown Whorl.

In only ten pairs of our identical twins do we find reversed asymmetry of any sort (either left-handedness, counter-clockwise hair-whorl, or both) in both twins of a pair. In eight of these cases (73, 25, 23, 87, 43, 38, 7, 27) both twins of a pair are left-handed or both have counter-clockwise hair whorl. It would seem natural to assume that all such pairs have been derived from zygotes, genetically left-handed. But what shall we do with the other two cases (13 and 72) in which one twin of each pair is plainly left-handed and the other clearly counter-clockwise in hair whorl? Since both of these indications are valid criteria of reversed asymmetry there seems no escape from the conclusion that these two pairs also are derived from genetically left-handed zygotes.

THE INCIDENCE OF ASYMMETRY REVERSAL IN OUR OWN COLLECTION OF TWINS.

When the present study began, the writer was keenly on the lookout for evidences of asymmetry reversal in identical twins. The expectation was that the more strikingly identical the twins were, the more evidence of asymmetry reversal would be present. Before the study was half over it seemed certain that this expectation was not to be realized. In fact, the very opposite of this appeared to be true, namely, that the least evidence of asymmetry reversal appears among those twins that are practically indistinguishable, while the twins that are less nearly identical show the most evidence of reversal of asymmetry.

In order to test out this conception, the writer tried to arrange the fifty pairs of identical twins in the order of their closeness of resemblance, including resemblances in features, height, weight, headsize, finger prints and palm prints. After this was done, Mrs. Blythe Mitchell, the one who has had the most intimate and prolonged acquaintance with the twins, was asked to rearrange the cases according to her impression as to the degrees of identity. On the whole, there was a very close agreement, no case being changed more than a few places up or down in the series. Using the photographs, we worked over all cases together and arrived at the arrangement shown in Table I., which is not intended to be exact, place for place, but certainly represents a real grouping in the sense that the first five pairs are more similar than the second five, the second five than the third, the ninth five than the tenth. Within groups of five the order might be more or less shifted as the criteria of resemblance are not entirely objective, but depend to a large extent upon one's judgment of degrees of facial resemblance. In the following table asymmetries in handedness, crown-whorl, and head dimensions are given for the fifty pairs arranged in fives, beginning with the most alike and ending with the least alike. In this table *R* and *L* indicate definite right- and left-handedness, *l* indicates partial left-handedness, *A* indicates ambidextrality; + indicates clockwise, or the common type of hair-whorl; and — indicates counter-clockwise hair-whorl. Double hair-whorls are indicated by (+ —).

TABLE I.

Serial No.	Twin.	Sex.	Handedness.	Crown Whorl.	Head Length.	Size Breadth.	Remarks.
62	A	♂	R	+	17.4	15.5	
	B	♂	R	+	17.2	15.2	
98	A	♀	R	+	17.7	13.9	
	B	♀	R	+	17.5	14.1	
63	A	♂	R	+	18.2	14.3	
	B	♂	R	—	18.1	13.9	
40	A	♂	R	(+ —)	17.85	15.0	
	B	♂	R	+	18.1	15.5	
3	A	♂	R	?	20.0	14.75	Negroes. Crown-whorl
	B	♂	R	?	19.7	14.3	could not be made out.
9	A	♀	R	—	17.0	13.6	A shows partial asymmetry reversal in crown.
	B	♀	R	+	17.7	13.7	
80	A	♀	R	+	18.7	13.5	
	B	♀	R	+	18.1	13.2	
67	A	♂	R	+	18.9	14.6	
	B	♂	l	+	18.7	14.1	B left-handed in wrist tapping.
55	A	♂	R	+	19.2	14.8	
	B	♂	R	+	19.1	14.4	
35	A	♂	R	—	18.55	15.0	A shows partial asymmetry reversal in crown.
	B	♂	R	+	18.55	15.1	
96	A	♂	R	—	18.10	13.9	
	B	♂	R	+	18.15	13.9	
* 73	A	♀	l	—	17.1	13.35	A, incompletely reversed crown; left-handed in finger tapping.
	B	♀	A	—	17.5	13.5	B, completely reversed in crown; nearly ambidextrous.
102	A	♀	R	—	17.8	15.0	
	B	♀	R	+	18.1	15.0	
* 25	A	♂	R	—	18.3	14.2	
	B	♂	R	—	17.9	13.9	
30	A	♀	R	+	18.8	13.7	
	B	♀	R	+	18.7	13.55	

TABLE I. (*Continued.*)

Serial No.	Twin.	Sex.	Handedness.	Crown Whorl.	Head Length.	Size Breadth.	Remarks.
* 23	A	♀	A	+	16.2	14.25	A more left-handed than B.
	B	♀	A	+	16.1	14.0	
94	A	♀	R	+	18.4	13.5	
	B	♀	L	+	18.15	13.2	
68	A	♀	R	−	17.45	14.25	
	B	♀	R	+	17.5	14.4	
49	A	♀	l	−	18.0	14.6	
	B	♀	R	+	18.2	14.1	
* 13	A	♀	R	−	17.0	14.5	
	B	♀	L	+	17.0	14.6	
78	A	♂	L	+	18.45	14.7	
	B	♂	R	+	18.40	14.7	
* 87	A	♂	A	−	19.95	13.9	A more decidedly left-handed.
	B	♂	A	+	19.7	14.0	
* 43	A	♂	l	−	19.6	15.7	Bats left-handed naturally.
	B	♂	l	+	19.1	15.3	Left-handed in finger tapping.
* 38	A	♀	l	−	19.4	15.5	Left in both wrist and finger tapping.
	B	♀	l	−	19.1	15.5	Left in finger tapping only.
79	A	♂	R	+	17.35	13.9	
	B	♂	L	+	18.1	13.3	
* 72	A	♂	L	+	19.15	14.5	
	B	♂	R	−	19.25	15.0	
99	A	♂	R	+	18.2	14.5	
	B	♂	R	+	18.25	14.5	
33	A	♂	L	+	18.05	14.9	
	B	♂	R	+	18.05	15.25	
53	A	♂	R	+	19.0	13.9	
	B	♂	L	+	18.8	13.4	
44	A	♂	L	+	18.5	14.3	
	B	♂	R	+	19.5	13.9	
2	A	♀	L	?	17.45	14.45	
	B	♀	R	?	17.95	14.35	
91	A	♀	R	+	18.0	13.8	
	B	♀	R	+	17.9	13.9	
100	A	♂	R	+	17.65	13.6	
	B	♂	R	+	17.2	14.0	

TABLE I. (*Continued.*)

Serial No.	Twin.	Sex.	Handedness.	Crown Whorl.	Head Length.	Size Breadth.	Remarks.
101	A	♂	R	+	18.6	15.5	
	B	♂	L	+	18.9	15.5	
70	A	♂	R	+	18.5	15.6	
	B	♂	L	+	18.1	15.3	
37	A	♂	R	+	19.15	14.75	
	B	♂	l	+	19.35	14.65	Slightly left-handed in wrist tapping.
34	A	♂	R	+	18.8	14.8	Inclined to be ambidextrous.
	B	♂	l	+	19.0	14.8	Slightly left-handed in finger tapping.
28	A	♀	R	–	16.8	13.8	
	B	♀	R	+	17.7	13.9	
* 7	A	♂	l	–	17.3	15.7	Strongly left-handed in finger tapping.
	B	♂	l	+	17.6	15.5	Strongly left-handed in finger tapping.
6	A	♀	R	+	17.5	13.9	
	B	♀	R	+	17.2	13.7	
97	A	♀	R	+	17.85	16.0	
	B	♀	R	+	18.0	16.0	
17	A	♀	R	+	17.85	14.45	
	B	♀	R	+	17.75	14.65	
14	A	♀	R	–	17.2	14.45	
	B	♀	R	+	17.45	14.45	
15	A	♂	R	+	19.65	14.6	Both ambidextrous as babies.
	B	♂	R	–	19.45	14.9	
69	A	♂	R	+	18.75	14.9	
	B	♂	R	+	18.9	14.3	
24	A	♂	R	+	17.45	14.65	
	B	♂	l	+	17.4	14.65	Slightly left-handed in playing marbles.
18	A	♂	R	+	18.6	13.8	
	B	♂	R	+	18.8	13.8	
* 27	A	♂	R	–	19.25	13.5	
	B	♂	l	–	19.3	13.7	Slightly left-handed in wrist and finger tapping.
41	A	♀	L	?	17.26	15.2	Hair whorl could not be determined.
	B	♀	R	?	17.6	15.6	
60	A	♀	R	+	17.6	14.1	
	B	♀	l	+	17.5	14.15	Slightly left-handed in finger tapping.

Handedness in Relation to Degrees of Resemblance.

In this table there are listed twelve pairs of twins one member of which is fully left-handed and, in addition to these, there are eleven cases that show partial left-handedness in one or both individuals of the pair. Besides the twenty-three cases showing some degree of left-handedness, there are two cases in which both members of the pair are classed as ambidextrous. Thus in exactly fifty per cent. of our pairs of identical twins there is some degree of left-handedness.

It is significant that the first case in the series to show complete left-handedness is seventeenth out of fifty. There are only two cases of partial left-handedness among the fifteen most strikingly similar set of twins, while some degree of left-handedness becomes the rule rather than the exception from the sixteenth to the end of the list.

Crown Whorl in Relation to Degrees of Resemblance.

The incidence of asymmetry reversal in crown hair whorl follows the same general lines as does left-handedness. In the first ten pairs there is but one case (No. 63, in third place) that shows true counter-clockwise hair-whorl. Two other cases (No. 9, in sixth place, and No. 35, in tenth place) show a mixed hair-whorl partly clockwise and partly counter-clockwise. There is also one case of a double hair-whorl, one whorl being clockwise, the other counter-clockwise (No. 40, in fourth place). The most frequent incidence of counter-clockwise hair-whorl in one twin occurs among the middle grade twins, neither the most alike or the most different. This is true also of left-handedness, and such a correspondence in the incidence of two forms of asymmetry reversal must have some real significance.

The Relation between Handedness and Head Size.

In the following study both left-handedness and counter-clockwise hair-whorl are taken to be equivalent criteria of either genetic or epigenetic reversal of asymmetry. For the present we shall omit from consideration the ten pairs of twins (Nos. 73, 25, 23, 13, 87, 43, 38, 72, 7 and 27) that were diagnosed as derived from zygotes genetically left-handed. These are starred in the list.

Before discussing the relation of head-size to handedness it should be said that there is undoubtedly some inaccuracy in the figures for head dimensions. Repeated measurements of the same head rarely give exactly the same result. Dahlberg has calculated that the average error in head measurements is about 0.5 mm. It seems probable that our own errors were at least as great as this, and probably greater. Hence differences of no more than 1 mm. may be ignored or considered as without significance.

Glance with me down the list of forty pairs of identical twins not previously diagnosed as derived from genetically left-handed zygotes. In all, there are seventeen pairs in which one twin may be classed as right-handed, the other left-handed, and in which there is a significant difference in head size. In thirteen of these pairs (63, 9, 67, 102, 68, 33, 53, 44, 2, 70, 28, 14 and 41) the right-handed individual, derived from the superior side of the embryo, has a distinctly larger head.

The four other cases (49, 79, 101 and 34) reverse this condition, the left-hander has the larger head, though case 34 is ambiguous in that one twin is slightly left-handed in tapping and the other nearly ambidextrous and may therefore belong with the list of ten diagnosed as derived from a left-handed zygote. The other three cases (49, 79 and 101) are valid exceptions. Let us consider these cases carefully. What would happen in the case of a genetically left-handed zygote if one of the twins underwent asymmetry reversal? Obviously the reversed twin would be a right-hander, and should have the smaller head. This interpretation appears to fit cases 49, 79 and 101. It would be strange if some cases such as these did not occur in view of the existence of genetically left-handed zygotes.

This hypothesis, that head size is correlated with handedness, may be checked still further by examining the ten pairs of twins diagnosed as derived from genetically left-handed zygotes. Of these, eight show a significant difference in head size. These eight cases deserve individual attention:

Pair No. 73.—This is a confusing case. Twin A shows left-handedness in finger tapping and has a partially reversed hair-whorl; twin B is practically ambidextrous in tapping and has a well-defined counter-clockwise hair-whorl, the only really positive indication of left-handedness present in the pair. This twin (B) has the larger head.

Pair No. 25.—In this pair both twins are right-handed and both have counter-clockwise hair-whorl. It is impossible to decide which of these has been derived from the superior side or whether they are derived from a right-handed or left-handed zygote, for the handedness and hair-whorl completely contradict each other.

Pair No. 23.—Both twins are ambidextrous, and both have clockwise hair-whorl. Twin A, with the larger head, is more nearly left-handed than B.

Pair No. 87.—Twin A, while ambidextrous, tends to be more left-handed than B, and has counter-clockwise hair-whorl; twin B is ambidextrous and has clockwise hair-whorl. Evidently A is the left-handed (superior) individual, and he has the larger head.

Pair No. 43.—Both twins are partly (probably natively) left-handed. Twin A has counter-clockwise hair-whorl, twin B clock wise. A, the more distinctly left-handed twin, has the larger head.

Pair No. 38.—Both twins are partially left-handed and both have counter-clockwise hair-whorl. A is left-handed in both wrist and finger tapping; B, only in finger tapping. A, the more left-handed, has the larger head.

Pair No. 72.—Twin A is strongly left-handed but has clockwise hair-whorl; twin B is right-handed but has counter-clockwise hair-whorl. It is impossible to say which individual should be diagnosed as from the superior side, since the two criteria seem to be of equal value. Of the two the reversed hair-whorl is somewhat safer as a criterion, and it happens that the twin (B) with the counter-clockwise hair-whorl has the larger head.

Pair No. 27.—Twin A is right-handed; B, slightly left-handed in wrist and finger tapping. Both have counter-clockwise hair-whorl. Twin B, the partially left-handed member of the pair, has the larger head.

All of these eight cases except pair 25, which is neutral, support the conclusion that the twin derived from the genetically superior side (the right side in these cases) of the embryo has the larger head.

One other class of cases remains to be dealt with, those in which a significant difference in head size exists without any complete asymmetry reversal in handedness or hair-whorl. There are nine such pairs (62, 3, 80, 55, 30, 6, 97, 69, 18). In all but two of these cases (3, 55, 30, 6, 97, 69, 18) one twin was definitely more right-handed than the other and the more right-handed individual has the larger head in all pairs. In pairs 62 and 80, both twins are equally strongly right-handed and offer neutral evidence. Instead of weakening the general theory, then, all of these cases, where varying degrees of difference in right-handedness but no true left-handedness occur, tend strongly to support it. There is beyond question a strong correlation between handedness and head size. With very few exceptions indeed, the twin having the larger head shows evidence of having been derived from the genetically superior side of the embryo; from the left-hand side in twins derived from zygotes genetically destined to form right-handers, and from the right side of zygotes destined to form left-handers.

Twinning and the Asymmetry Mechanism.

The data just presented have given rise to a theory that seems to rationalize for the first time the peculiar incidence of reversal of asymmetry in twins. It is well known that in some groups of animals, notably those characterized by a striking degree of determinate cleavage, bilateral symmetry and asymmetry are established in the undivided zygote before or at the time of the first cleavage. In those forms, on the other hand, that show a strong tendency toward indeterminate cleavage, notably the vertebrates and echinoderms, symmetry and asymmetry are not definitely fixed until considerably later in development. The writer's work (Newman, 1924) on asymmetry reversal in the starfish indicates clearly that asymmetry is fixed before the time of gastrulation, for no reversal of asymmtry could be induced in embryos older than late blastulæ.

There are also indications among the vertebrates that asymmetry is established prior to or during gastrulation. Thus in the nine-banded armadillo, the only case of twinning among mammals where the stage at which twinning occurs is definitely known, it has been found that the first step in the twinning process usually precedes the period at which symmetry and asymmetry are established and that the second step in twinning takes place during the process of the establishment of the axis of symmetry. By analogy, we may infer that twinning in man takes place in close association with, and possibly as an aberration of, the process of establishing and fixing the relations of symmetry and asymmetry in the embryo.

Now, since no biologic processes takes place with the same clock-like precision in all specimens, we may suppose that the twinning act in some cases is consummated during relatively early stages of the establishment of symmetry and asymmetry, and that in other cases it is established later. In the cases in which twinning occurs relatively late, the establishment of a single bilateral individual may have gone so far that complete twinning is impossible. This is probably the case in all partial twinning, resulting in conjoined twins and double monsters. In such twins one of the most striking features is the occurrence of profound reversal of asymmetry, as expressed in more or less complete *situs inversus viscerum.*

If then, we may assume that conjoined twins with the most extreme reversal of asymmetry in the inferior component, represents one end of the series of twins, it is natural to assume that the opposite end of the series is represented by cases in which twinning is consummated before any asymmetry is fixed. In such cases the twins would be derived from two equivalent primordia which had not yet been differentiated into right- and left-hand sides. When, later, asymmetry comes to be established in these two genetically equivalent and still undifferentiated embryos, it should follow the same course in both and each should develop the same asymmetry as the embryo would have done had it not undergone twinning. Thus, if the original embryo was genetically a right-hander, two right-handed twins should result; similarly, if the original embryo was genetically a left-hander, two left-handed twins should result—a condition not uncommon among twins, but hitherto unexplained. In such twins we would expect a high degree of same-sided asymmetry in such details as palm and finger prints, ear shape, dentition, handedness, hair-whorl, etc. Moreover, since the two twins are derived from two primordia that have not yet become differentiated as right- and left-hand components, the two resulting twins would be expected to be very strikingly similar, more similar than would be twins separated after asymmetry had been more or less fixed in the embryo from which they are derived.

Thus the earlier twinning occurs with respect to the establishment of asymmetry, the more similar should be the resultant twins and the less should they show such evidences of reversal of asymmetry as left-handedness and counter-clockwise hair whorl. This explains why these criteria of asymmetry reversal are rarely present in the most strikingly similar twins and are increasingly common among identical twins that are less similar.

If this theory be sound, and there is much evidence in its favor, we have discovered another mechanism, not classifiable as environmental, that operates to make identical twins different. This factor, the asymmetry mechanism, may be the main, if not the only, factor responsible for observed differences between identical twins reared together. Consequently it would be quite unsafe to infer that any differences between such twins are due to differences in environment or in training. On the other hand, once we have established the average degree of difference between identical twins reared together, we should be able to use this as a base line in determining to what extent, in cases of identical twins reared apart, the differences in environment have operated to increase the physical or mental difference.

This theory goes far to explain why some, but not all, pairs of twins show left-handedness and counter-clockwise hair-whorl in one twin of a pair; why there should be occasional cases in which both twins of a pair are left-handed or have counter-clockwise hair-whorl; why there should be various degrees of incomplete asymmetry reversal as the result of separation of twins prior to complete establishment of asymmetry. The establishment of asymmetry is a progressive process and takes some time to become fully fixed. Hence we may expect to find that twinning early in the process will result in little if any signs of asymmetry reversal in one of the twins, and that twinning occurring late in the process will result in extensive reversal of asymmetry in one of the components.

In brief, this theory seems to clear up many if not all the formerly baffling asymmetry situations found in twins. It lacks experimental confirmation, but this must be so from the nature of the material. Yet the data themselves almost speak out the theory of their own accord.

SUMMARY.

1. Reversal of asymmetry in monozygotic twins expresses itself in varying degrees, ranging from complete *situs inversus viscerum* in conjoined twins to left-handedness or counter-clockwise hair-whorl in separate twins.

2. There are two kinds of handedness: genetic and epigenetic. Genetic right- and left-handedness have about the incidence, respectively, of dominant and recessive allelomorphs. Epigenetic left-handedness (or in genetic left-handers, right-handedness) results from twinning, the inferior side having an asymmetry opposite to that of the superior side.

3. Arranging fifty pairs of identical twins in the order of their closeness of physical resemblance, it is found that there is very little evidence of asymmetry reversal among the most similar twins, while the less similar twins show a high degree of it.

4. Clockwise hair-whorl has about the same incidence as right-handedness, and counter-clockwise hair-whorl that of left-handedness.

5. Varying degrees of partial left-handedness and of ambidextrality are revealed by tapping tests.

6. Ten pairs of identical twins show asymmetry reversal in both members of a pair and are therefore diagnosed as derived from genetically "left-handed" zygotes; three pairs showing asymmetry reversal in but one twin should probably be classed as "left-handers"; the remaining thirty-seven pairs are believed to be derived from right-handed zygotes.

7. There is a very close correlation between head size and handedness. The twin derived from the superior side of the embryo nearly always has a significantly larger head.

8. The reason why many but not all identical twins show asymmetry reversal in one twin is that the epigenetic establishment of asymmetry takes place sometimes before and sometimes after twinning. If it takes place before twinning the twins will show

a high degree of asymmetry reversal; if it takes place after the twinning the twins will both show the same asymmetry and be in other respects more alike than when the establishment of asymmetry precedes twinning; if it takes place during the twinning process the twins will show varying degrees of asymmetry reversal in one individual and varying degrees of close resemblance in physical and mental characters.

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BIOLOGICAL BULLETIN

SEX DIFFERENTIATION IN GONADS DEVELOPED FROM TRANSPLANTS OF THE INTERMEDIATE MESODERM OF *AMBLYSTOMA*.

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INTRODUCTORY AND HISTORICAL.

From his studies on parabiotic twins in *Amblystoma punctatum* Burns ('25) is led to the conclusion that in this species there may occur a complete reversal of sex previous to the period of sex-differentiation. Embryos joined in pairs in early stages should, by the laws of chance, be combined in the proportion of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀. Instead of this expected ratio, Burns obtains exclusively one-sexed pairs, in the proportion of 44 ♂♂ to 36 ♀♀. This result he is inclined to interpret as a 1 : 1 ratio. Having no evidence that the two-sexed pairs had been eliminated through selective mortality, Burns postulates that half the pairs reared must have been, originally, ♀♂ combinations; in these pairs, from a condition of near-equilibrium as regards sex, one or the other sex, he assumes, had eventually gained the ascendency, so that at sex-differentiation the gonads of the two members of the pair were identical. Since the sex-ratio found was approximately 1 ♂♂ : 1 ♀♀, Burns infers that there can be no prepotency constantly favoring either male or female, since in this event a 3 : 1 ratio favoring the prepotent sex would be expected.

The more recent studies of Witschi ('27) on frog embryos joined in parabiosis show that in these amphibia the early sex reversal assumed by Burns does not occur. Witschi finds in 56 pairs the following combinations: ♂♂, 16 pairs; ♂♀, 17 pairs, with 7 of the females undergoing sex-reversal; ♀♂, 10 pairs, with 4 of the females undergoing sex-reversal; ♀♀, 13 pairs. This approximates very closely the expected ratio of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀, and shows conclusively that there could have been no sex-reversal previous to the time of sex-differentiation. From the fact that in many of the two-sexed pairs the females were found undergoing sex-reversal, while a female united with a male undergoing reversal was never found, Witschi concludes that the male is always dominant in the sex-reversal which finally occurs. Though he believes that the independent sex-differentiation in the individuals of genetically two-sexed pairs favors the theory of localized sex-differentiators ("lokalisierte Innenfaktoren," probably comparable to Spemann's "Organisatoren"), he states that in the later sex-reversal of the female of the pair, "the coöperation of hormones is not improbable."

The method of parabiosis used by Burns and Witschi has certain obvious disadvantages. If used with a species in which the zygotic sex-determination can be completely reversed previous to sex-differentiation, as is possibly the case in *Amblystoma*, there can be no certainty regarding the original state of any one-sexed pair examined after sex-differentiation has taken place. In drawing conclusions as to the occurrence of sex-reversal in these one-sexed pairs, one must depend entirely upon the sex-ratio obtained. Further, if the death rate among pairs joined in parabiosis is high, the possibility of a selective mortality cannot be entirely eliminated, even though evidence in favor of it may be scanty or lacking. Hence there is no absolute proof that the one-sexed pairs found at sex-differentiation were not all of this character genetically at the time they were joined; the proof of sex-reversal, therefore, remains inconclusive.

The method of parabiosis is relatively advantageous if used with a species in which an early reversal of sex does not occur (as *Rana sylvatica*; Witschi, '27). In such a species, pairs preserved at a suitable period in development would show the actual progress of sex-reversal in one member of the pair. If, however, the reversal becomes complete, all pairs killed at later periods would be found to be one-sexed. Although sex-reversal could be confidently asserted for a species of this type as a result of the disappearance of the two-sexed condition observable in younger pairs, the identity of any of the originally mixed pairs could be established in adult animals only with great difficulty if at all.

In the spring of 1926 the writer undertook to transplant the intermediate mesoderm of *Amblystoma* from one embryo to a latero-ventral site in another in order to determine the fate of the primordial germ cells included in such grafts. Among the seven embryos surviving the implantation was one in which at forty-four days after operation the germ cells of the graft were found to have given rise to a gonad of considerable size (Humphrey, '27). This suggested the possibility that such grafts, if allowed to develop until after the period of sex-differentiation of the host, might be found to contain gonads which had likewise undergone sex-differentiation. The donor serving as the source of the transplant, and the host into which it was engrafted, though selected at random long before sex-differentiation had occurred, must in many cases be unlike in sex. Since the donor could be reared, its sex could be determined from the gonad it possessed, and since donor and host were not joined, the sex-differentiation in either could not be influenced by the other, except in so far as the graft might be able to modify the sex-differentiation of the host. If, then, after sexual differentiation the gonad of the graft were found to agree in type with that of the host regardless of the sex of the donor, the fact of an early sex-reversal would be established beyond question. If, on the other, hand, the gonad of any graft differed in type from that of the host, agreeing with that of the donor, it would show conclusively that sex-reversal previous to sex-differentiation had not occurred. By the method of grafting, therefore, it seemed possible to obviate certain difficulties inherent in the method of parabiosis. The donor furnishing the graft would undergo sex-differentiation according to the factors present in the egg at fertilization; its gonad could be compared directly with the gonad developed in a transplant removed during the germ-layer stage and grown in a host of the opposite sex. Conclusions as to the occurrence of sex-reversal, therefore, could be drawn from comparison of structures rather than by reasoning from sex ratios in which the factor of selective mortality might possibly be involved.

MATERIAL AND METHODS.

The removal of the intermediate mesoderm (preprimordia of gonad and mesonephros) of *Amblystoma* and its implantation into another embryo is a relatively simple operation, the technique for which has been outlined elsewhere (Humphrey, '27). During the operating season of 1927, 180 such implantations were carried out. The graft always included a large part of that region of the intermediate mesoderm in which it had been found that primordial germ cells develop (*i.e.*, the territory of the seventh to the seventeenth somites, approximately); in addition it included parts of the adjacent axial and lateral mesoderm, together with the overlying ectoderm.

Following operation, the host receiving the transplant and the donor furnishing it were reared to the age of fifty days or over—*i.e.*, until after the beginning of morphological sex-differentiation. At autopsy of the host the graft derivatives were found, as a rule, attached to the inside of the ventral or lateral body wall. In the donor, at autopsy, the gonad was always very small or entirely lacking on the right, the side from which the transplant was invariably taken in the embryo.

RESULTS.

Of 180 pairs (donor and host) only 49 or 27 per cent. of the total, were reared to the age of 50 days or over. This, however, does not indicate an actual mortality of 73 per cent. in the grafted animals, since 25 additional hosts were reared to the age of 50 days or more, although the donors which had furnished them transplants had died in early stages of development. Several hosts were also killed before reaching the age of 50 days, in order to study the development of the gonad and other structures in the graft; these hosts were always those of pairs from which the donor had already died from operative injury or other causes. In all, 74 grafts were recovered after sex of the host had become distinguishable. Of these grafts, 40 contained a gonad, the sex of which was determinable with a fair degree of certainty in 33 cases. In the remaining grafts the gonad was small with few germ cells and no features permitting it to be classified as either ovary or testis.

Unfortunately for this study, the majority of the gonads which developed were in homoplastic transplants in *Amblystoma jeffersonianum*. In this species, instead of the expected 1 : 1 sex-ratio, the animals reared in the laboratory in 1927 were in the proportion of 56 females to 19 males, essentially a ratio of 3 : 1. As a result of the predominance of females, donor and host were both of this sex in an excessive number of cases. In only two instances were donor and host unlike in sex and in these, unfortunately, the gonad of the graft was in each case of somewhat atypical structure due to unfavorable environmental factors.

To the writer's knowledge a sex-ratio such as the one here reported for *Amblystoma jeffersonianum* has not been previously recorded for this species. Whether it is to be explained on the basis of a selective mortality among operated animals, or whether it is due to an induced reversal of sex in certain males resulting from nutritive disturbance or other alteration of environmental factors, or whether an excess of females is a normal condition in this species or at least in its local strain, cannot be positively stated. It is worthy of note that in *Amblystoma maculatum* (*punctatum*) reared in the laboratory under identical conditions and after similar operative procedure, the sex-ratio is apparently quite normal. The collection of large numbers of *A. jeffersonianum* larvæ from local ponds and a study of their sex-ratio has not been possible. The few specimens picked up near ponds after metamorphosis have been found to be females in the great majority of cases.

A second feature of interest noted particularly in this species is the occurrence of spermatocyte stages in the testes of males 60 to 80 days of age. This cannot be due to the presence of a graft furnished by a female, since spermatocytes are no more frequent in hosts than in donors. Though Burns ('25) makes no mention of spermatocytes in *A. maculatum* of similar age, the writer has encountered such stages occasionally in this species as well as in *A. jeffersonianum*. In the latter, however, they occur in a higher percentage of the males examined, and usually in greater numbers than in *A. maculatum*. In neither species, were the spermatocytes found in stages later than the pachytene condition of the heterotypic prophase. Though the presence of heterotypic prophases in males renders these stages of little value as a criterion of sex when considered alone, it may be noted that their number in the male is small as compared with the number of other germ cells, and that they were not found in the diplotene or later stages characteristic of the oöcytes of amphibian females.

Of 56 *Amblystoma maculatum* reared in the laboratory in 1927 30 were females and 26 males. These numbers give an approximation of the expected 1 : 1 ratio. In this species, however, the majority of the transplants used were furnished by very young donors (stages 21 to 25 [1]) and but few gonads developed. In only two cases in which the sex of the donor was known to differ from that of the host was a gonad present in the graft. In one of these two the gonad was small and of the indifferent type, while in the second it was of a type combining features of both ovary and testis.

From the above it may be seen that relatively little evidence bearing upon the problem of sex-reversal was obtainable from grafts the donors of which had survived to sex-differentiation. But in several cases in which the donor had died before reaching this period, the transplant furnished by it was found to contain a gonad differing in sex type from that of the host in which the graft had developed. In these cases it would appear that donor and host must have been unlike in sex, but that the gonad of the graft had differentiated in a fashion determined by the organization of the transplant previous to its isolation from the donor. These cases may now be described in some detail.

No. 211.—Transplant from *A. jeffersonianum* of stage 29 implanted in *A. maculatum* of stage 25. The donor died 18 days after operation. The host, killed 61 days after operation, proved to be a female. A section of the ovary is shown in Fig. 1. The central ovarian cavity is well developed, and the germ cells are peripheral in position. Their nuclei are largely in the heterotypic prophase stages characteristic of the early urodele ovary, although few in number or lacking in the testis, as a rule, until a much later period of development. The graft removed from this host included a fairly large gonad of testicular type (see Fig. 2). No central cavity is present. The germ cells are somewhat uniformly scattered through the organ, intermingled with numerous smaller cells which constitute the 'sex cords' (anlagen of duct system), and the stromal and sustentacular elements of the testis. The germ cells are all in spermatogonial stages; heterotypic prophases are entirely lacking.

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[1] The stages referred to throughout this paper are those of Harrison's series of standard stages.

No. 284.—Transplant from *A. maculatum* of stage 30 implanted in host of same species and stage. The donor was killed by the host 37 days after operation. The host, autopsied 58 days after implantation of the graft, is a female. Although sex-differentiation had but recently occurred, the ovary has the characteristic central cavity and peripheral oöcytes with nuclei in heterotypic prophase (see Fig. 3). The gonad found in the transplant is a pear-shaped testis attached by a stalk to the surface of the graft mesonephros. It lacks the central cavity characteristic of the ovary, and shows the more uniform distribution of germ cells typical of the young testis (see Fig. 4). No heterotypic prophases are present, all germ cells being in spermatogonial stages.

No. 244.—Transplant from *A. jeffersonianum* of stage 31, implanted in host of same species and stage. Donor presumably devoured by host at about 31 days after operation. The host, autopsied 61 days after operation, is a male; a section of one testis is shown in Fig. 5. As is frequently the case in males at this stage of development, the testes of this animal show a few cells in the spermatocyte stage, but the germ cells are distributed in the fashion characteristic of the testis, and no central cavity is present. For comparison with the testis of the host a section of the gonad of the graft is shown in Fig. 6. This gonad must be interpreted as an ovary in an early stage of sex-differentiation. Although no central cavity is yet present, the germ cells are arranged in a layer around the periphery of the gonad and are for the most part oöcytes in earlier stages of the heterotypic prophase. By comparison of Fig. 6 with Figs. 1 and 3 (ovaries of fairly early stages of differentiation) it will be readily appreciated that this graft gonad is ovarian in nature. The differences between it and the graft gonads of Figs. 2 and 4 (testes) are clearly evident from the photographs, and need no further comment.

The three cases above described show clearly that a gonad developing in a graft need not agree in sex type with the gonad of the host. It may be logically inferred that in these three cases the sex-differentiation of the graft gonad was determined by the organization in the implanted mesoderm previous to its removal from the donor embryo.

In a few cases in which both donor and host lived until after sex-differentiation and were found to be of unlike sex, a gonad was present in the graft. These cases, however, are less satisfactory than the preceding, since the gonad of the graft is either in an early stage of sex-differentiation or is of atypical structure. Three such cases will now be described.

No. 207.—Transplant from *A. jeffersonianum* of stage 29 implanted in *A. maculatum* of stage 25. The host, killed 61 days after operation, is unquestionably a male, although a few germ cells in heterotypic prophase are found in one of the testes. A section of the testis is shown in Fig. 7. The donor, a female, was not killed until 78 days after operation. The gonad shown in Fig. 8 is therefore more advanced in development than the testis of Fig. 7. The gonad found in the graft is small and in an early stage of sex-differentiation. Although no central cavity is present, the germ cells tend to take a peripheral position. Of the 95 germ cells present, 38 are in early stages of the heterotypic prophase. Considering all its structural features, this gonad should be classed as an ovary. In the peripheral arrangement of its germ cells, and in the high proportion of these cells found in heterotypic prophase, it is clearly similar to the gonad of the donor rather than to that of the host.

No. 190.—Transplant from *A. jeffersonianum* of stage 33 implanted in host embryo of same age and species. The host, killed 64 days after operation, is a female (see ovary in Fig. 10). The donor, killed at the same age as the host, is a male (see Fig. 11). The gonad of the graft is atypical in structure in that an unusual amount of stroma is present, in the form of a mucous type of connective tissue (Fig. 12). It may nevertheless be classed as testis rather than ovary. The germ cells, though frequently included in the covering epithelium, are predominantly scattered through the central part of the organ. No central cavity is present. Sex cords (anlagen of duct system of testis) are recognizable as groups or strands of smaller cells, in some sections extending a third or more of the length of the gonad. The germ cells are for the most part spermatogonial in type, only three or four of the several dozen present being in heterotypic prophase, and none of these having the characteristics of growing oöcytes. Though of atypical structure, this gonad cannot be considered as undergoing transformation from testis into ovary. Aside from the abundance of mucous connective tissue, its structural features are clearly similar to those of the testis in the donor. Atypical gonads of the same general appearance may develop in grafts from a male donor implanted in a male host. The peculiarities of structure exhibited are therefore due, probably, to the action of local environmental factors rather than to the activity of sex hormones secreted by the gonads of the host.

No. 188.—Transplant from *A. jeffersonianum* of stage 33 implanted in host of same age and species. Both donor and host were killed 64 days after operation. The host is a female, the donor a male (see Figs. 13 and 14). The gonad of the graft is an atypical structure difficult to classify (see Fig. 15). Neither typical ovarian cavity nor testicular duct system is recognizable. The germ cells are predominantly peripheral in location, although frequently scattered or in masses deeper within the stroma. In one instance a mass of germ cells lies in a cavity, with no apparent attachment to other tissues of the gonad; these cells show marked degenerative changes. The cells at the periphery of the gonad frequently exhibit a grouping or 'nesting' comparable to that of young oöcytes in a normal ovary. Though for the most part in heterotypic prophase (several hundred such cells must be present) these germ cells seem never to progress beyond the pachytene stage. If the gonad were actually ovarian, some few at least of these cells might be expected to pass through the diplotene stage and then enlarge as growing oöcytes. This has been found to occur in those atypical gonads which have developed in grafts from female donors. In this gonad, however, no growing oöcytes are present, numerous pyknotic and fragmenting nuclei indicating the degeneration of the germ cells during the pachytene stage rather than their continued development.

While it might appear at first glance that the features exhibited by this gonad have resulted from the action of the hormones of the host, it is highly probable that many of its peculiarities are referable to the growth potentialities of the implanted tissue as modified through local environmental influences. The donor furnishing this particular transplant exhibits an unusual number of spermatocytes in its one (left) gonad. Four such cells may be recognized in the section shown in Fig. 14 (at left). Presumably the tissue implanted possessed the potentiality for developing a gonad in which unusual numbers of heterotypic prophases would have appeared precociously, even without an endocrine stimulus from a female host. As to local environmental conditions, it may be noted that the gonad was attached by a very delicate fold of tissue, and was apparently poorly vascularized. The latter condition alone would be unfavorable to the development of a gonad of normal histological structure.

In addition to the graft gonad above described (No. 188) two other specimens exhibit features which might possibly be interpreted as modifications due to the action of sex-differentiating hormones. In one of these the graft gonad consists of a central core of testicular character overlaid by a cortex ovarian in type. This structure resembles the modified testes described by Burns ('28) as resulting from the action of ovarian hormones. The position of this particular graft in the body of the host, however, is such that some of the primordial germ cells of the host may actually have entered into the make-up of the graft gonad. If this be the case, this structure must be regarded as a 'mosaic' gonad derived from two preprimordia of unlike sex-potentialities rather than as a testis undergoing sex-reversal due to the endocrine influence of a female host. It is significant that graft gonads developing in sites sufficiently far ventral to exclude the possibility of actual contribution of host germ cells generally show no indication of sex-reversal (see Figs. 2, 4, and 6).

Among those cases in which only the host survived until the period of sex-differentiation are seven in which the gonad of the graft agrees in type with those of the host. While a reversal of sex in these few cases cannot be positively excluded, it is rendered exceedingly improbable by the fact that in five other cases the gonad of the graft is of opposite sex from those of the host. Examples of this latter group have already been described (Nos. 211, 284, and 244; Figs. 1 to 6).

Discussion.

The outstanding feature of the results described in the preceding pages is the apparently independent sex-differentiation of the gonads which develop in grafts. Although in one or two cases such a gonad has been modified in a fashion suggesting an influence from sex hormones of the host, in no case is a complete early reversal of sex clearly indicated. So far as can be determined from cases in which the sex of the donor is known, the primary sex-differentiation in the gonad of the graft always proceeds in a fashion determined by the sex of the donor. In five cases in which the sex of the donor is not known, the gonad is of opposite sex from that of the host. In four of these cases, gonads with the features characteristic of a testis have differentiated in grafts implanted in female hosts, while in the fifth an ovary has developed in a graft implanted in a male.

It is difficult to reconcile these findings with the conclusions reached by Burns ('25) from his studies on the sex of parabiotic twins in *Amblystoma*. Burns finds that the sex of the two members of any pair is always the same. From this he is led to infer that complete reversal of sex has occurred in one member of all two-sexed pairs, such reversal being accomplished before sex differences in the gonads become morphologically distinguishable. He assumes that when embryos of unlike sex are joined in parabiosis there results a condition of close balance or unstable equilibrium, which is broken if one animal of the pair gains a slight advantage, presumably through earlier or more abundant output of sex-differentiating hormones. All hormones being mingled in the blood stream, and neither sex being constantly prepotent, either the male or the female hormone may become dominant. Such domination being established before the onset of morphological sex-differentiation, the phenomena of this period will be identical in the two members of any parabiotic combination, or essentially so. The twin which has undergone reversal thus differentiates directly without first exhibiting the sexual characters to be expected from its genetic constitution. Under these conditions, a reversal cannot be detected by study of developmental stages of the gonads but must be inferred from the absence of two-sexed pairs after morphological differentiation has been completed, unless it be assumed that all such pairs have been eliminated through a selective mortality.

In discussing his results, Burns considers the possibility that a 'selective' mortality has operated to eliminate all heterogeneous (male-female) pairs, permitting only homogeneous pairs to survive. While this explanation cannot be positively rejected, Burns regards the occurrence of a selective mortality as highly improbable. Although the death rate among his operated animals is very high (about 77 per cent.), he believes that it is possible to explain it without postulating a physiological incompatibility of the sexes so profound as to induce the death of all two-sexed pairs. Witschi ('27) has demonstrated that no such incompatibility exists in the frog, since he finds the expected number of mixed pairs at metamorphosis of his parabiotic animals. If we assume that among Burns's experimental animals there was likewise no selective mortality eliminating mixed pairs, we are forced to conclude that parabiosis induces an early sex-reversal in one member of every two-sexed pairs.

If sex-reversal in parabiotic twins in *Amblystoma* be assumed to have occurred in the manner postulated by Burns, it would be logical to expect a reversal of sex in the gonad of a graft implanted in a host of opposite sex from that of the donor. The bulk of the transplant is small compared with the entire body of the host, and the gonad to which the graft gives rise is but a fraction of the size of the host's own gonads. Under these conditions there should exist no state of near-equilibrium as regards sex. If sex-differentiating hormones are produced previous to morphological sex-differentiation, those of the host should always, from their greater abundance, be able to dominate the differentiation of the gonad in the graft;[2] the latter, therefore, should always agree in type with the gonads of the host. Yet the gonad of a graft is clearly able to develop as ovary in a male host, or as testis in a female. In none of my animals could sex-reversal be demonstrated as having preceded the primary sex-differentiation.

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[2] The gonad of the graft is often somewhat retarded in development as compared with those of the host, possibly, in some cases, because of inadequate nutrition. Such retardation of its development should favor modification of the graft gonad by the gonads of the host, assuming that sex differentiating hormones are poured into the circulation when the gonads reach a certain stage in their differentiation.

Since the extent to which a hormone may modify an embryonic structure probably depends in part upon the period of development at which it is introduced and the time during which it is allowed to act, these conditioning factors may well be compared for parabiotic twin and graft.

In Burns's experiments, *Amblystoma* embryos were joined in parabiosis at about stage 28 of Harrison's series. In my own experiments many of the grafts were implanted at this or even earlier stages. In none of the cases considered in this paper was either donor or host more advanced in development than stage 34 at the time of operation. In neither the parabiotic twins at the time of union nor in the host receiving an implant has the blood yet begun circulation. While it is probable that the blood streams of embryos joined in parabiosis are in communication from the time the circulations of the two first become established, my observations indicate that the graft becomes vascularized at a correspondingly early period in its development. In short, the sex-modifying influence of the host upon the graft should be exerted fully as early as the influence of an embryo upon its parabiotic twin, assuming that this influence is mediated through the activity of substances transported by the blood.

As regards the actual time elapsing between operation and autopsy, the advantage appears to lie with the parabiotic twins. Burns states that among the pairs of his series even the best did not show sex-differentiation until seventy days, while the general average required considerably longer (eighty to ninety days) for sex to become clearly distinguishable. In my own animals sex was usually determinable without difficulty at fifty days after operation. The longer indifferent period in the parabiotic twins doubtless results chiefly from growth retardation due to difficulties in feeding. In any event, it greatly increases the period over which one animal is subjected to the influence of the other before morphological sex-differentiation occurs. Possibly in this prolonged indifferent period the physiological state of the gonads in one animal may be so altered through the influence of its opposite-sexed twin that at the time morphological sex-differentiation finally occurs the gonads of the two animals differentiate in identical fashion. In my own experimental animals the shorter indifferent period may be insufficient to effect such a physiological reversal in the gonad of the graft, which in consequence differentiates as determined by the genetic constitution of the donor. In the parabiosis experiments of Witschi the indifferent period (in *Rana sylvatica*) is likewise short, which may possibly explain the fact that sex-reversal of the female follows rather than precedes the primary morphological differentiation of the gonad.

It is also possible that conditions attendant upon development of the graft may render it less subject to hormone influence from the host than is a parabiotic twin to the influence of its mate. Since the graft usually becomes well vascularized, however, it would seem that the nutritive materials and hormones of the host's blood stream should be as readily available for the gonad of the graft as for the host's own gonads. As has been stated before, sex-differentiating hormones of the host should be but little diluted by antagonistic hormones secreted in the graft. Moreover, the removal of the graft from its natural environment in the donor while in a germ-layer stage and its implantation into an essentially foreign situation should, if anything, disturb the action of local factors affecting sex-differentiation, and facilitate the modification of this process through hormones produced by the host. It would seem that in a graft the developing gonad has been removed from both the endocrine and environmental influence of the donor and subjected to the influence of the host in a far more complete fashion than the gonads of one parabiotic twin can be brought under the influence of the other embryo of the pair.

From comparison of the conditions acting upon parabiotic twin and graft, we may conclude that two, at least, possibly have significance in determining the difference in the results obtained. First, the greater time required for morphological sex-differentiation in parabiotic twins may permit an influence of one animal upon the other such as would not be possible in the case of a graft gonad differentiating in from half to two-thirds of the same period. Secondly, the fact that in one case (parabiosis) the gonad has remained undisturbed in the organism, while in the other its preprimordium has been implanted in an ectopic situation in another individual, may possibly explain the different way in which it reacts preceding or during sex-differentiation.

The results obtained by the writer in *Amblystoma* are not without parallel from experimental work on other vertebrates. Willier ('27), from his study of the differentiation of chick gonads implanted in the chorio-allantoic membranes of either male or female hosts is led to the conclusion that "the course of sex-differentiation in the chick embryo is apparently not determined by the action of sexual hormones circulating in the blood stream." He believes that "hormonic sex-differentiating factors of the host embryo are either absent, or if present, they are ineffective in the modification of the engrafted sexual glands." Witschi ('27*b*) reaches similar conclusions from one of his latest studies on sex-differentiation in *Rana temporaria*. He finds that the implantation of a large graft of adult frog testis in tadpoles of this species does not "exert the least influence upon the larval and early post-larval development of the gonads." In both frog and chick, therefore, the indifferent gonads are found to undergo their primary sex-differentiation apparently unmodified by sex hormones from outside sources. In cattle, too, recent studies may be interpreted as showing that even when the chorions of two-sexed twins are fused at a very early period, the gonad of the female co-twin first begins to differentiate as an ovary, and only later undergoes modifications leading to the production of the characteristic free-martin gonad (Lillie, '23; Bissonnette, '28).

That the vertebrate ovary in situ may be modified in its development subsequent to its primary sex-differentiation is apparent from the studies of Lillie ('17) and others on the free-martin, or from the cases of sex-reversal in parabiotic frogs reported by Witschi ('27*a*). That these same gonads would have undergone a comparable modification if implanted as grafts in a host of the opposite sex has not been actually demonstrated. According to Willier, no modification of engrafted gonads of the chick is demonstrable after a period of nine days on the host embryo. It is conceivable, however, as Willier states, that the transplantation of the embryonic sexual glands into chicks after hatching might yield different results than when these same glands are implanted on the membranes of embryonic hosts. Greenwood ('25) has reported the development of spermatic tubules in grafts of the left ovary taken from chicks two to four days after hatching and implanted in young chicks of the same age. It would appear probable, therefore, that isolation and implantation of a gonad (or its preprimordium) do not necessarily prevent the modification of that gonad through the action of sex-hormones of the host: *i.e.*, there remains possible an inhibition of growth, or an induction of growth, in those parts (as for example the medullary cords of the bird's ovary) which have retained their embryonic capacity to react in a specific fashion to growth stimuli.

The grafts described in this paper were in no case left implanted in the host for a period longer than seventy days. Although in none of the grafts recovered had the gonad undergone a complete reversal of sex previous to its primary differentiation, it is possible that in one or two cases it had undergone some slight modification which might be ascribed to the action of sex hormones of the host. Whether a complete reversal of sex might have occurred had the graft remained implanted for a longer period is problematic. From grafting experiments recently reported by Burns ('27) it is evident that sex-reversal in the gonads of *Amblystoma* is not complete even after periods of from fifty to seventy-six days in a host of the opposite sex. Burns transplanted gonads from larval stages, just before and just after the beginning of morphological sex-differentiation, into older larvæ in which sex-differentiation was more advanced. Since several of the grafts showed an admixture of the characteristics of the two sexes, it is possible that a complete reversal of sex might eventually have been effected.

Whether or not complete reversal of sex in *Amblystoma* may occur subsequent to morphological sex-differentiation, a reversal of sex preceding this period does not appear to be effected in gonads developed in grafts, when such grafts are implanted in an ectopic situation, such as the latero-ventral body wall. Whether implantation of the graft into its normal site would insure reversal of the gonad as postulated for animals joined in parabiosis still remains a question. The writer now has in progress an extensive series of experiments to test this point.

Summary and Conclusions.

1. An area of mesoderm which included the preprimordium of the gonad was transplanted from one *Amblystoma* embryo to another at stages 21 to 34. Such transplants, when taken from donors older than stage 25, gave rise to a gonad in a high percentage of cases. This gonad was ectopic in position, being attached to the inside of the lateral or ventral body wall, and was always far smaller than the normal gonads of the host.

2. Morphological sex-differentiation occurred in the grafts at from fifty to sixty days after implantation. All grafts were removed and fixed within seventy days. In several cases, gonads of testicular type were recovered from female hosts. In two cases gonads of ovarian type were found in grafts implanted in males.

3. In two cases in which donor and host were of opposite sex the gonad of the graft was modified in such fashion as to suggest an influence from sex hormones of the host. In no case, however, was the sex of the graft gonad completely reversed previous to the period of morphological sex-differentiation.

4. It may be concluded that gonads developed in ectopic grafts of the gonadal preprimordia undergo their primary morphological sex-differentiation according to the organization of the graft at the time of its removal from the donor.

5. If sex-differentiating hormones are produced by the host previous to morphological sex-differentiation, they are apparently incapable of bringing about reversal in the gonad of the graft. The possibility of reversal at a later stage of development is not excluded, since no grafts were allowed to develop for periods longer than seventy days.

6. The failure of the gonad in a graft to undergo sex-reversal previous to its morphological differentiation is in marked contrast to the complete reversal which appears to occur in parabiosis (cf. Burns '25).[3]

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[3] Studies completed while this paper was in press indicate that the graft ovaries of Figs. 6 and 9 possibly owe certain features of their structure to the action of the testicular hormones of the host. These studies show that the developing ovary is readily modified if subjected to the continued influence of a testis resident in the same host, and that one of the first perceptible indications of this modification is the absence of the characteristic central ovarian cavity. These studies will be reported in a separate paper.

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PLATE I. EXPLANATION OF FIGURES.

All figures on this plate are photomicrographs. Magnification 145 ×.

FIG. 1. Ovary of host No. 211, *Amblystoma maculatum*, killed 61 days after implantation of graft at stage 29. The central ovarian cavity is well developed, and the germ cells peripheral to it are chiefly oöcytes in heterotypic prophase. Compare with Fig. 2.

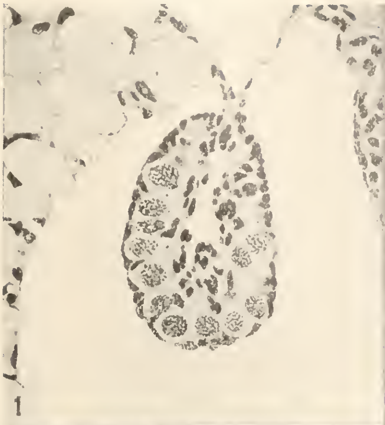
FIG. 2. Testis of graft recovered from host No. 211. Note the absence of a central cavity. The germ cells are uniformly distributed, and none are in heterotypic prophase. Compare with the ovary of the host (Fig. 1). This testis was attached to the body wall by a slender stalk not included in this section.

FIG. 3. Ovary of host No. 284, *Amblystoma maculatum*, killed 50 days after implantation of graft at stage 30. Ovarian cavity, peripheral arrangement of germ cells, and abundance of heterotypic prophase stages, as in Fig. 1. Compare with graft gonad of Fig. 4.

FIG. 4. Testis of graft recovered from host No. 284. Note absence of central cavity and heterotypic prophases, and the uniform distribution of the germ cells. Compare with the ovary of the host in which this testis developed (Fig. 3). The slender stalk attaching the testis to the mesonephros of the graft is not included in this section.

FIG. 5. Testis of host No. 244, *Amblystoma jeffersonianum*, autopsied 61 days after implantation of graft at stage 31. This gonad exhibits the scattered arrangement of germ cells and the absence of a central cavity noted in the testes of Figs. 2 and 4. Compare with graft gonad shown in Fig. 6.

FIG. 6. Ovary of graft recovered from host No. 244. Although the central cavity is not yet developed, the germ cells are peripheral in position and are for the most part in heterotypic prophase. This gonad thus resembles an ovary (see Figs. 1 and 3) rather than the testes of the host from which it was recovered (see Fig. 5).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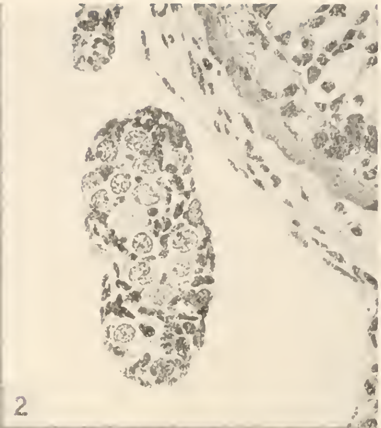
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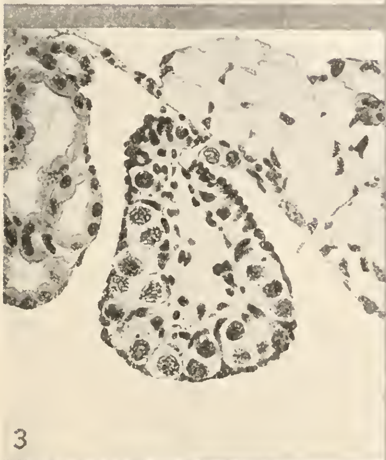
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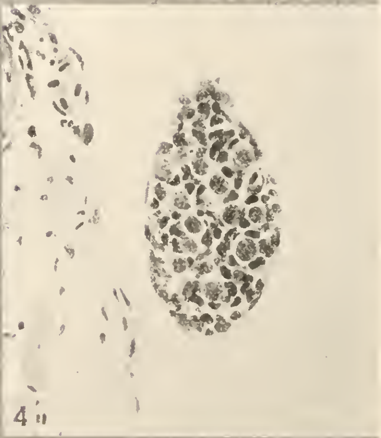
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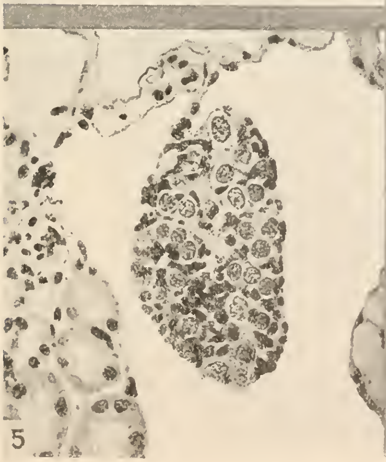
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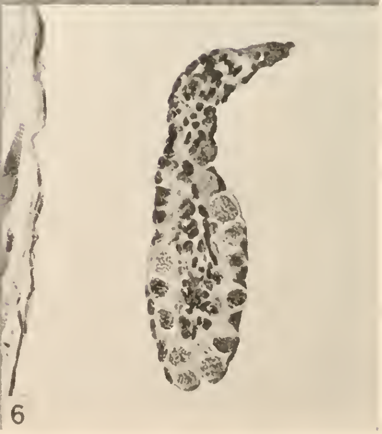
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R. R. HUMPHREY.

Plate II. Explanation of Figures.

All figures on this plate are photomicrographs. The magnification is 121 × except for Figs. 8 and 9, in which it is 162 ×.

Fig. 7. Testis of host No. 207, *Amblystoma maculatum*, killed 61 days after implantation of graft at stage 25.

Fig. 8. Left ovary of donor No. 207, *Amblystoma jeffersonianum*, killed 78 days after furnishing graft (at stage 29) for implantation in host No. 207. Due to the age at which this animal was killed, the ovary is advanced in development as compared with those of Figs. 1 and 3.

Fig. 9. Gonad of graft recovered from host No. 207. Though retarded in its differentiation, this gonad is apparently an ovary, since its germ cells are peripheral in arrangement, and a large proportion of them are in heterotypic prophase stages. Compare with Figs. 7 and 8.

Fig. 10. Ovary of host No. 190, *Amblystoma jeffersonianum*, killed 64 days after implantation of graft at stage 33.

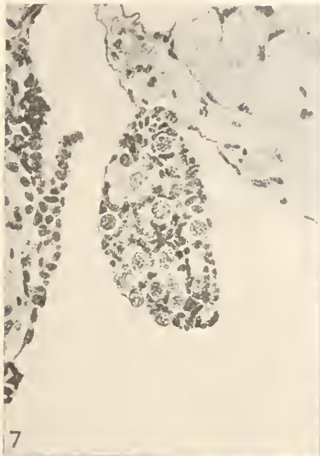
Fig. 11. Left testis of donor No. 190, *Amblystoma jeffersonianum*, killed 64 days after furnishing graft for implantation in host No. 190.

Fig. 12. Gonad of graft recovered from host No. 190. Though atypical in structure, due to the presence of mucous connective tissue, this gonad is apparently a testis. No central cavity is present, the germ cells are scattered, and but very few of them are in heterotypic prophase. Compare with gonad of donor (Fig. 11).

Fig. 13. Ovary of host No. 188, *Amblystoma jeffersonianum*, killed 64 days after implantation of graft at stage 33.

Fig. 14. Left testis of donor No. 188, *Amblystoma jeffersonianum*, killed 64 days after furnishing graft for implantation in host No. 188.

Fig. 15. Gonad of graft recovered from host No. 188. It lacks a central cavity, but has its germ cells predominantly peripheral in position, and frequently in groups or "nests" as in the ovary. Although many of its germ cells are in heterotypic prophase, this is true also of the testis of the donor. This gonad is possibly a testis modified by reason of its development in a graft in a female host.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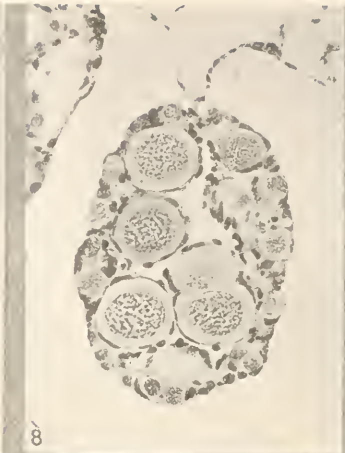
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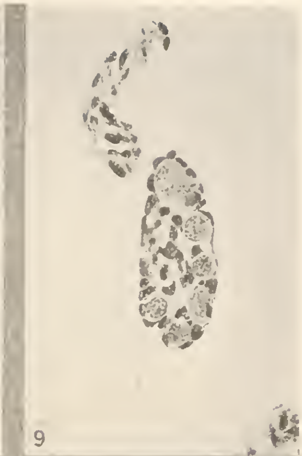
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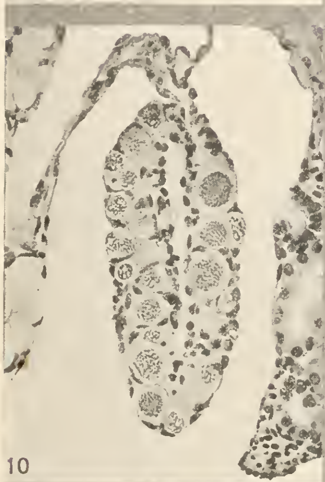
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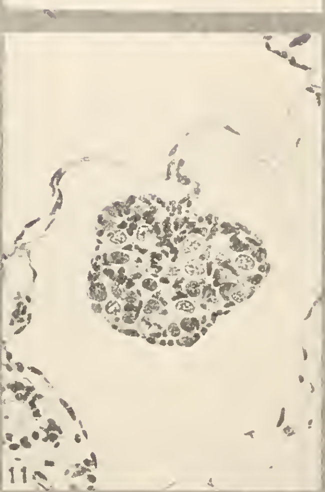
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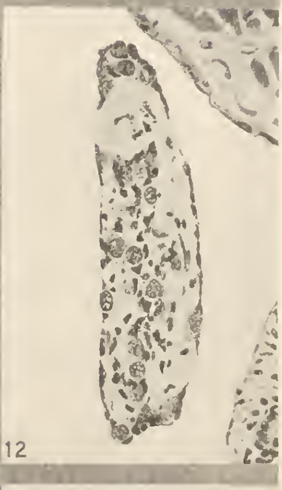
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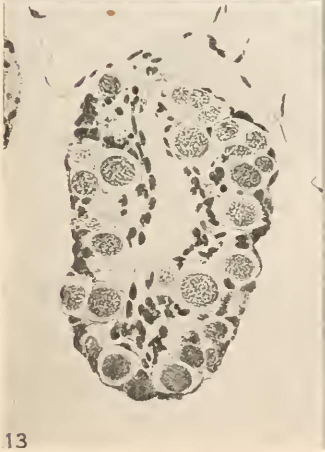
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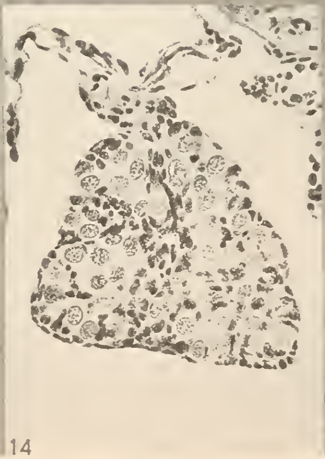
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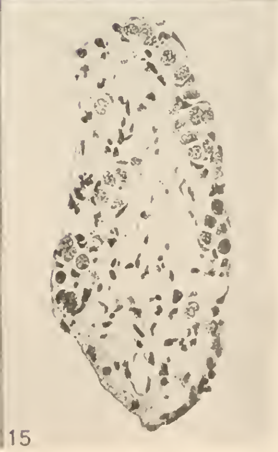
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R. R. HUMPHREY.

ON THE PROPERTIES OF THE GONADS AS CONTROLLERS OF SOMATIC AND PSYCHICAL CHARACTERISTICS.

XI. Hormone Production in the Normal Testes, Cryptorchid Testes and Non-living Testis Grafts as Indicated by the Spermatozoön Motility Test.[1]

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I. Introduction.

Advances in the study of the internal secretions are very often largely dependent upon the development of successful indicators for the substances concerned. Since the studies of Brown-Sequard, innumerable attempts have been made to increase our knowledge of the internal secretions of the sex glands, and indeed vast stores of information have been accumulated through these investigations. The chief difficulty in many of these attempts and especially in attempts to obtain the hormone principle in extractions, has been the lack of an applicable indicator of the substances sought for isolation.

A tremendous step forward in the study of the female hormone was the demonstration of the details of the œstrous cycle as indicated by vaginal smears first by Stockard and Papanicolau ('17) in the guinea pig and later by Long and Evans ('22) in the rat. By the vaginal smear method, one is enabled to determine the presence or absence of substances concerned with the regulation of the œstrous cycle. With such a useful indicator, the advances made in the study of the internal secretions of the ovary have been indeed marked.

On the male side, however, the situation has been a less happy one from the standpoint of real advancement. In some species

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[1] This investigation has been aided by a grant from the Committee for research in problems of sex of the National Research Council; grant administered by Prof. F. R. Lillie.

of the bird, notably certain breeds of the domestic fowl, the male feather pattern, behavior, and head furnishings have afforded a good criterion of testicular presence and activity though many details were insufficiently known until of late to make conditions as well understood as was needed. The extensive work of Domm ('27) on the brown leghorn breed has given a much greater appreciation of the many pitfalls that present themselves in this field (for a review of the extensive literature on this subject, see Domm's paper).

When we approach the study of the internal secretions of the gonads in the male mammal, however, a careful analysis will show the marked absence of useful criteria to indicate the activity of the testis hormone, operating over limited periods of time. It is true that there is the sex impulse, supposedly entirely under the control of the internal secretions of the testicles, but many things lead us to believe this supposition to be erroneous. A castrated male theoretically should lose its attraction for the female, but I have repeatedly utilized guinea pigs castrated at 30 days of age as testers for the period of female acceptance for some months after castration. Stone ('27) has recently reported that young male rats castrated at the age of three months will continue to copulate with females for periods of four, five and even eight months. And it is reported that the eunuch, though castrated early in life, will years afterward have not only an attraction toward the female but experiences a degree of satisfaction in this association.

It is likewise true that the growth of the penis, seminal vesicles (when present), prostate, etc., are to a large extent dependent upon the internal secretions of the testis but not only have the variations in such structures proven so great as to make an assay of a given experimental procedure difficult and often impossible, but also, if castrations are made on adult animals, to be followed by such procedures as testis transplantations, injections or other possible approaches, the question of the condition of these structures as representing a balance between postoperative regression or possible stimulation from the materials or conditions utilized often presents insurmountable barriers.

Such other indicators for testicular internal secretions as individual body weight, body length, fat deposition, hair coat, and pugnacity as have been utilized by other workers serve often to mislead the investigator due to the lack of specificity of the indicator (for further criticisms of this phase see my papers, '21 and '22).

During the course of a study of the physiology of the scrotum or its heat regulating effects on the generative tissues of the testis (Moore, '24*a*, *b*; '26, '27, and '28; Moore and Quick, '24) a possible, fairly satisfactory indicator for the internal secretions of the testis in the differential survival of the capacity for motility of epididymal spermatozoa was discovered by accident. The same conditions were also discovered by Benoit ('26) a little earlier, in the course of his beautiful work on the histology and cytology of the epididymis. The application of this "spermatozoön-motility" test for the testis hormone has been under investigation in this laboratory for longer than three years. The principle of the test may be expressed in details for the guinea pig.

When both testes of an adult guinea pig are removed from the animal, leaving the inferior portion of the epididymides, containing their millions of spermatozoa, in the normal scrotal position, one finds that the spermatozoa gradually lose their capacity for motility when these are suspended in physiological saline solution. A lessened capacity for motility is evident within a few days after testis removal and seldom can one see any degree of motility in the spermatozoa after a period of twenty-three days following the operation. However, if instead of removing both testes, one is allowed to remain normal, the single, opposite, epididymis will contain spermatozoa that show motility when suspended in saline solution for a period of sixty-five to seventy days (Moore, '28). The difference between the 23 days retention of the capacity for motility when both testes have been removed and that of 65 days when one testis has remained, has been proven to be an expression of the internal secretion of the testis (for further details of this reaction see Moore, '28).

While it is freely admitted that the spermatozoön motility reaction has many limitations we have found it very useful and it will continue to be useful until a better hormone indicator has been discovered. In the following pages a few items of information with respect to its usefulness will be presented.

II. Hormone Production by Normal Testes.

Utilizing the spermatozoön motility reaction in the guinea pig as described above, I have attempted to study hormone production in the normal testis to learn more concerning its action upon the life of spermatozoa when it is removed from the animal through castration at varying periods during the possible life of the mature germ cell. It has been indicated, for example, that under the influence of the full hormone compliment of one testis, the spermatozoön life, as shown by its capacity to exhibit motion in physiological saline solution, gradually wanes until after approximately 65 to 70 days it no longer responds to this stimulus. Should we, for example, wish to supply the hormone by testis transplantation or by injection of material supposed to contain it, we should be able, if possible, to test the effect in the shortest time within which the reaction will indicate any effect. What influence, therefore, does hormone supplied by a testis *in situ* exert when it acts for ten, fifteen or twenty days? The following procedure will present the method employed.

Young adult guinea pigs are operated under ether anæsthesia through a low mid-abdominal incision and one testis withdrawn into the field of operation. The testis is carefully separated from the inferior pole of the epididymis (tail portion), the internal spermatic vessels are ligated and the testis, along with the head and body of the epididymis removed. The remaining (inferior) portion of the epididymis, connected with its vas deferens, is then carefully replaced in the scrotum—a necessary precaution (see Heller, '29). Ten or fifteen days later the opposite normal testis is removed entire through a scrotal incision. At selected intervals after the second operation, the animals are sacrificed, the isolated epididymis finely hashed with scissors in a small quantity of physiological saline and examined immediately with the microscope for spermatozoön motility. To properly express gradations in motility * signs have been employed in which the normal movement is expressed by ****; the barest vibratile movement on the part of a few spermatozoa (perhaps 1 in 10,000 will contract weakly with little or no translation) is rated *. Where no movement can be detected the observation is designated o.

Table I. will serve to illustrate the observations on the motility of spermatozoa obtained from the isolated epididymis of animals whose normal testis was allowed to remain for 10, 15, 20, 30, and 40 days after the epididymis to be tested had been isolated.

TABLE I.

UNILATERAL EPIDIDYMAL ISOLATION; OPPOSITE TESTIS REMOVED SUBSEQUENT TO ISOLATION AS INDICATED IN DIFFERENT GROUPS (GUINEA PIG).

Animal.	Epididymal Isolation.	Killed.	Days after Isolation.	Motility.	Excess Life beyond 23 Days Attributed to Hormone Effect.
			(delayed removal 10 days)		
517	1–31	3–1	30	*	7 days
518	1–31	3–3	32	*	9 days
520	1–31	3–5	34	o	
521	1–31	3–5	34	o	
			(delayed removal 15 days)		
406	6–10	7–11	31	**	8 days
407	6–10	7–14	34	***	11 days
409	6–10	7–20	40	o	
409	6–10	7–20	40	**	17 days
410	6–1	7–24	44	**	20 days
411	6–11	7–29	48	o	
412	6–11	7–29	48	o	
413	6–11	7–29	48	o	
414	6–11	7–29	48	o	
415	6–11	7–29	48	*	25 days
			(delayed removal 20 days)		
441	10–21	11–29	39	*	16 days
442	10–21	11–29	39	*	16 days
443	10–21	12–3	43	*	20 days
444	10–21	12–3	43	*	20 days
445	10–21	12–6	46	o	
446	10–21	12–6	46	o	
			(delayed removal 30 days)		
450	10–25	12–8	44	**	21 days
447	10–25	12–17	5	*	30 days
451	10–25	12–20	56	*	33 days
452	10–25	12–24	60	o	
			(delayed removal 40 days)		
469	12–3	1–25	53	*	30 days
472	12–3	1–25	53	o	
470	12–3	1–27	55	o	
473	12–3	1–27	55	*	32 days
474	12–3	1–31	59	o	

To understand the observations recorded it must be remembered that when an epididymis is isolated from its testis and both testes are removed from the animal, the spermatozoa contained within an epididymis retain their capacity to show motility for a period of 23 days; this we may call the basic life period and realize that they will live for this period without any hormone being produced by the testicle.

From the table it can be seen that under "delayed removal 10 days" the spermatozoa were observed to show motility for 32 days or nine days longer than expected, had both testes been removed at the time of epididymal isolation. We see, therefore, that the hormone supplied by the normal testicle for a period of ten days before its removal, actually extended the life of the spermatozoön nine days. Similarly, hormone supplied for fifteen days extended the retention of the capacity for motility for a similar length of time (actually slightly longer since in animal 415 a few sperm were seen to move slightly on the 48th day after isolation or an increase of 25 days above the basic 23 days expected). When the normal testis was allowed to remain 20 days after epididymal isolation, motile capacity was increased 20 days beyond what it would have been had both testes been removed at the first operation. Hormone supplied by the normal testis for 30 days permitted retention of spermatozoön motility up to 56 days or 33 days longer than the natural life without hormone being supplied. A hormone supply from the normal testis for forty days increased the sperm life by little more than thirty days. But it must be remembered that as we add to the length of time after operation we gradually approach the natural limits of spermatozoön life even with a full compliment of hormone; this limit is 65 to 70 days. We could not therefore expect the relative progressive effectiveness to continue much beyond a 40-day normal testis retention because of the approach to the maximum period of persistence of sperm under a continuous hormone influence. In an earlier paper I have emphasized that even utilizing the greatest possible care in the selection of standard animals for operation and in doing the operation itself, there is an individual animal variability that cannot be eliminated; at best we can only make an approach toward quantitative relationships.

Utilizing the basic 23 days as the approximate maximum of retention of the capacity for motility on the part of spermatozoa when no hormone is being supplied (and in scores of observations I have never observed motility for periods above 23 days) we see that one can actually detect the influence of the testis hormone when it is supplied for only ten days. Due to the individual animal variability, I would consider attempts to read reactions more finely as decidedly unprofitable. In fact, to err on the safe side, I have arbitrarily chosen to regard any tested substance or condition involving gonads as negative unless the capacity for motility is retained for thirty days or longer.

It is of interest to examine the data of the above table with reference to what they may tell us of hormone production and storage. When a ten day hormone supply by the normal testis shows an effect of ten days in the reaction, and likewise when spermatozoön motility is extended 15, 20, and 30 days beyond the basic expectations in conditions wherein the testis was present for 15, 20 and 30 days after epididymal isolation, one must conclude, I believe—(1) that hormone secretion is a continuous process and (2) that the hormone is not stored within the body. When hormone is supplied by the normal testis, for 10 days, the reaction indicates an effect for the same length of time.

III. Hormone Production in Cryptorchid Testes.

It has long been known that man or the domestic mammals may experience a failure of testicular descent into the scrotum. Such animals, although always sterile, nevertheless possess their full compliment of secondary sex characters; they are spoken of as Cryptorchid individuals. The undescended testes of such animals have long been known to lack a germinal epithelium; the gametogenetic function of the testis is deficient but its internal secretory effects are not visibly diminished. It is now known that a normal testis removed from the scrotum and confined within the abdomen very rapidly loses its germinal epithelium and assumes within a month or two, almost identical characteristics to those testes that have never descended. The cause of the degeneration of testes confined within the abdomen has been found to be the warmer environment of the abdomen and the function of the scrotum has thus been seen to be that of a local thermoregulator (for details of this work see Moore, 1924*a*, 1924*b*, and 1926; Moore and Quick, '24).

Regarding the amount of hormone produced by such a cryptorchid testis, occurring normally or artificially made, little is known. It could be assumed perhaps that a smaller quantity of hormone might be required to produce or to maintain the secondary sex characteristics than would be required to maintain completely normal male conditions. Lipschutz and his co-workers have maintained that in the rabbit 1 per cent. of the normal amount of testicular tissue is sufficient to maintain all the secondary sex characters ('22). On the other hand, it has been assumed by some investigators that any condition leading to an "Apparent increase in interstitial cells" whether by testis transplantation, X-rays, vasoligation (this latter is the basis of the contentions underlying the ideas of the Steinach rejuvenation hypothesis) or any other means, presages an increased production of hormone. The argument proceeds from the assumption—First, that the apparent compensatory hypertrophy of interstitial cells is real (see discussion Moore, '24*a*; Bascom, '25); and second, that the hormone is produced exclusively by the Leydig cells. Some authors have gone so far as to speak of castrated males, bearing testis grafts containing appreciable amounts of interstitial tissue, as "supermales," an implication that I consider without any basis of fact.

In order to gain any new information possible regarding the quantitative aspects of hormone production by cryptorchid testes, I have utilized the guinea pig in the following manner: Young adult guinea pigs have been operated so that one testicle was removed from the scrotum into the abdomen and the inguinal canal closed to prevent scrotal redescent. Four months, and five months, later a second operation was made to isolate the normal epididymis from, and to remove, the normal testicle. We thus have an isolated epididymis with its spermatozoön content to use as a test for the hormone produced by the opposite degenerate four or five months experimental cryptorchid testis.

Table II. is a record of observations made on eleven animals in which one testis was confined to the abdomen for four months and upon six animals where hormone supply came from a testis confined in the abdomen for five months.

Table II.

Unilateral Cryporchidism Four Months; Normal Testis Removed from Epididymis, and Effect of Four Months Cryptorchid Testis on Spermatozoön Motility Determined.

Animal.	Isolation of Normal Epididymis.	Killed.	Days since Isolation.	Motility.	Wt. of Cryptorchid Testes.*
393	Oct. 13	Nov. 25	43	***	Not recorded
394	Oct. 13	Dec. 5	53	**	0.26 gms.
402	Oct. 17	Dec. 8	52	***	0.424 "
396	Oct. 13	Dec. 12	60	*	0.21 "
397	Oct. 13	Dec. 12	60	0	0.175 "
398	Oct. 13	Dec. 17	65	*	0.158 "
400	Oct. 13	Dec. 17	65	0	0.130 "
403	Oct. 17	Dec. 21	65	*	Not recorded
404	Oct. 17	Dec. 26	70	*	0.170 gms.
416	Oct. 17	Dec. 31	75	*	0.255 "
417	Oct. 17	Jan. 7	82	0	0.205 "

Unilateral Cryptorchid Five Months; Subsequent Treatment similar to Above.

475	Apr. 28	June 26	59	**	0.153 gms.
476	Apr. 28	June 30	63	*	0.13 "
477	Apr. 28	July 6	69	0	Not recorded
478	Apr. 28	July 6	69	*	Not recorded
479	Apr. 30	July 12	73	0	Not recorded
481	Apr. 30	July 12	73	*	0.095 gms.

* Testis weight, without epididymis or fat body.

It will be seen from Table II. that spermatozoa within the isolated epididymis have been observed to retain their capacity to show movement on proper stimulation for seventy to seventy-five days. Since the normal testicle, actively carrying on its spermatogenetic activity supplies only sufficient hormone to permit the sperm to live for the same length of time, we must conclude that the hormone producing capacity of a non-gametogenetic, degenerative, or cryptorchid testicle as measured by the spermatozoön motility test is the equivalent of the normal testis.

Figure 1 is introduced to show the histological character of the testicle after abdominal confinement (experimental cryptorchidism) for a period of seven months; the microphotograph shows a portion of a section from the testicle of animal No. 481. This testicle had been confined within the abdomen for a period of five months, when the epididymis of the opposite testicle was isolated for the subsequent spermatozoön-motility test and the normal testicle removed from the animal. Reference to Table II. will serve to recall that the test epididymis contained a few living spermatozoa 73 days after epididymal isolation and these few exhibited very weak motility on suspension in saline solution. When the animal was sacrificed on the 73d day after epididymal isolation the testicle had been confined in the abdomen for a period slightly longer than seven months. The weight of the organ, after removal of its attached epididymis, was 0.095 grams. Since the average weight of eight normal testicles, without the epididymis, removed from similar sized animals and at the same time of the year, was 1.7 grams (1.34 minimum wt.—

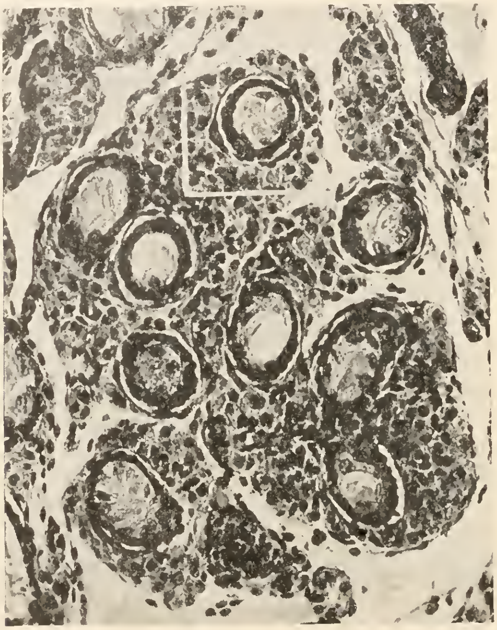

FIG. 1. Photomicrograph of portion of 7 months cryptorchid testicle (animal no. 481) showing shrunken seminiferous tubules separated by interstitial tissue.

2.06 maximum) it will be appreciated that the weight of this cryptorchid testis represents 2.8 per cent. of the total testicular weight of the normal animal. Had the epididymides been included in this weight the percentage of the normal testicular weight represented by this cryptorchid testis would have been considerably less; the spermatozoön and secretion mass within the normal epididymis being very much greater than the slight fluid content of the cryptorchid epididymis. It can be concluded, therefore, that the cryptorchid testis representing 2.8 per cent. of the normal testicular mass was producing sufficient hormone to maintain the life of spermatozoa in the isolated epididymis for the same period as would the hormone produced by two normal testes.

Figure 1 shows that the seminiferous tubules of the seven month cryptorchid testis were very much reduced in caliber and consisted of a basement membrane, somewhat thickened, and a few Sertoli nuclei and reticulum; the tubules were rather widely separated by interstitial tissue. Fig. 2 is a drawing of the tubule marked off by white lines at the upper part of Fig. 1. The thickened basement membrane is more clearly shown and the character of the contents of the tubule indicates an absence of any germ cells; the nuclei that are visible are believed to be Sertoli nuclei. Careful microscopic study has failed to bring to my attention any cell that appeared different from those represented in this figure and it is for that reason that I believe no

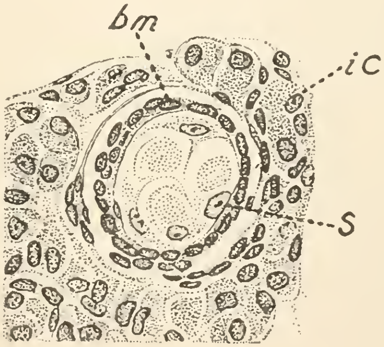

FIG. 2. Drawing of tubule marked off by white lines in Fig. 2. *bm*, basement membrane; *ic*, interstitial cell; *S*, Sertoli nucleus.

germinal cells were present in this testis, at the time of its removal.

IV. Hormone Production by Testis Grafts.

The question of the function of testis transplants must of necessity be considered under at least two categories: (1) The function of grafts which have become successfully incorporated into the host organism and remain as living masses of testicular tissue, and (2) the function of such masses of testis tissue transplanted into various parts of the host organism, which by reason of host resistance to the transplant or because of too great a mass of tissue for vascularization, dies and is resorbed or sloughed out of the incorporation bed often with pronounced suppuration. It is known that living testis grafts can be obtained and that they will function. This question, along with the presentation of personal observations, has been reviewed by me at some length (Moore, '26). The question of the function of testis tissue transplanted into a host organism under conditions that have been so unfavorable as to prevent its retention and growth has been dealt with most usually by the clinician. For various reasons many cases of transplantation in man of human testis tissue or testicular tissue from another mammal such as the ram, boar, monkey or deer, have been done. The effects reported are so all-embracing that discredit of all effect is engendered (for discussion of this work see Moore, '26). In general it may be repeated that the effects reported have been expressed in terms of the subjective feelings of the patient—whether he may feel better after remaining quietly in bed for a week or longer after the operation; or whether after the suggestions and discussion of the question and the anticipations of the operation and its outcome, he has a greater desire for coitus; or whether the patient feels that he can walk more sprightly or feels that he can climb a stairs two steps at a time instead of the customary pre-operation one step. In short, such evidence is worthless from the scientific point of view.

In order to study by objective means the question of the function of such non-living testis transplants, I have utilized the guinea pig as the experimental animal and the spermatozoön motility test as an indicator of effectiveness. In an earlier paper

(Moore, '28) I included a few observations then at hand and have since given additional attention to the problem. The method employed, in brief, is the bilateral isolation of the epididymides along with removal of the testes from the animal. The two testes removed were replaced immediately in an especially prepared subcutaneous incorporation bed made by tunneling under the skin, with some destruction of skin musculature and a general scarification of the particular region. Each testicle, cut into two parts, was placed in a separate implantation bed, one on each side of the mid-ventral line of incision.

In addition to the subcutaneous transplantation of the animal's own two testes at the time of epididymal isolation, a few cases of multiple transplantations were studied with the idea that perhaps a small amount of hormone might be liberated from the introduced tissue which if introduced more than once would conceivably show a greater effectiveness. Accordingly at the time of bilateral epididymal isolation, two, one-fourth testes, were introduced subcutaneously at the time of the first operation as well as on the 3d and 5th day following. In each animal, therefore, six transplantations were made, the aggregate amount of tissue transplanted being one and one half testes. The observations on four such experiments are recorded in Table III*B*.

Since an arbitrary choice of thirty days after operation for effectiveness to be registered was made, animals were sacrificed close to this period for the study of the spermatozoön content of the isolated epididymides. From section II. it will be seen that motility of spermatozoa at this time would indicate the equivalent of effectiveness of the normal testis present for seven to ten days. Too much uncertainty surrounds the application of this test to make it profitable to attempt readings at an earlier date.

Table III. presents some of the observations recorded.

Among the nineteen animals whose isolated epididymides were studied for spermatozoön movement between the 25th and 36th day after autoplastic transplantation of two testes, only two animals have shown any movement of spermatozoa and in each case (animals No. 320, No. 456) observed on the 31st day the motility was the weakest possible for a positive reading. Amid

TABLE III.

A. THE EFFECT OF NON-LIVING TESTIS GRAFTS ON SPERMATOZOÖN MOTILITY (GUINEA PIG).

Animal.	Date—Operation.	Date—Killed.	Days after Operation.	Motility.
364	4- 6-27	5-12-27	36	0 many non-mot. sperm
365	4- 6-27	5-12-27	36	0
370	4- 8-27	5-12-27	34	0 " " " "
371	4- 8-27	5-12-27	34	0 " " " "
372	4- 8-27	5-12-27	34	0 " " " "
373	4- 8-27	5-12-27	34	0 " " " "
320	11-22-26	12-23-26	31	* (1 in 1000 weak mot.)
436	10-15-27	11-15-27	31	* (1 in 1000 weak mot.)
435	10-15-27	11-15-27	31	0
347	3- 8-27	4- 7-27	30	0 many non-mot. sperm
376	4-15-27	5-15-27	30	0 " " " "
377	4-15-27	5-15-27	30	0 " " " "
439	10-20-27	11-19-27	30	0 " " " "
440	10-20-27	11-19-27	30	0 " " " "
437	10-20-27	11-19-27	30	0 " " " "
455	10-26-27	11-25-27	30	0 " " " "
457	10-27-27	11-25-27	29	0 " " " "
458	10-27-27	11-25-27	29	0 " " " "
453	10-25-27	11-19-27	25	0 " " " "

B. MULTIPLE GRAFTS AFTER BILATERAL EPIDIDYMAL ISOLATION.

Animal.	Transplantation Days after Epididymal Isolation.	Killed, Days after Testis Removal.	Motility.
553	1st, 3d, 5th	30	0
554	1st, 3d, 5th	30	0
555	1st, 3d, 5th	30	*
549	1st, 3d, 5th	32	0

the field of millions of spermatozoa, here and there, an individual cell could be seen to show a weak contraction, perhaps a weak vibratile movement every thirty seconds; a rough estimate of 1 in 1000 was made to give a relative notion of the quantity of spermatozoa capable of movement. In contrast to this, eleven animals observed a shorter period of time after operation (25 to 30 days) were all negative; no spermatozoön movement could be seen. One animal (No. 555) receiving six transplantations of one fourth of one testis at three different operations subsequent to testis removal, showed a few spermatozoa capable of weak movement on the 30th day after operation, whereas two others on the same day and one on the 32d day after operation failed to show any spermatozoa capable of exhibiting movement despite the fact that quantities of normal looking sperm were present.

These results show, therefore, that subcutaneous transplantation of testes provided such a small amount of hormone (if any at all) that its effect was less than the effect of a normal testis remaining *in situ* for seven to ten days after operation.

It is difficult or impossible, as pointed out above, to prevent individual animal variation. Whether the two positive readings on the 31st day are to be explained on this basis of more virile spermatozoa or as an indicator of some hormone effect cannot be stated. But since all operations were done alike, and equivalent masses of tissues transplanted, it would seem as if animals sacrificed earlier (between 25 and 30 days) would have given as strong or a stronger reaction than these three. In any event, should we attribute the results to hormone production and express it as a positive effect of the transplantations, the mildness of the reaction would still be evident. At best it is a questionable indication of hormone production.

The transplanted tissue reactions have been characteristic in all cases. A few days after transplantation the graft site, considerably removed from the line of skin incision, is swollen and decidedly reddened; the elevation caused by the transplanted tissue, at first scarcely visible, becomes approximately the size of a pigeon's egg or larger. It is typically an inflammatory reaction. Ten days after operation the swelling may be almost as large as three days after transplantation and an active pus discharge may be noted. In many cases, the pus spreads toward and escapes through the healing incision but in many cases erosion of the skin over the site of transplantation may provide an escape for the discharge through the new opening. Pus is often seen exuding from such areas up to the termination of the experiment (30 days). In some cases the transplanted mass has so completely sloughed by the termination of the experiment as to be invisible excepting as the site of transplantation may be marked by scar tissue. In other cases small encapsulated masses of pus are present.

It is evident from these observations and considerations that a mass of testicular tissues undergoing autolysis after transplantation gives little or no evidence of having liberated hormones into the organism.

V. Discussion.

Our three years experience with the spermatozoön-motility reaction as an indicator for the testis hormone have increased our confidence in the test as a dependable objective test for hormone production. Readily admitting that it lacks several desirable qualities to make it entirely adequate for many different approaches to the subject it must still be recognized as a valuable means of studying hormone production.

Relative to our interests here under discussion, we realize for the first time that the hormone produced by mammal testes is not stored within the body of the organism and the internal secretions of this organ are thus brought into line with such other organs producing internal secretions as parathyroids, pituitary, ovary, etc. In the ovarian follicle it has been realized that a temporary storage, perhaps at the site of production, does occur, but that the body does not ordinarily store it up for future release is shown by the failure of recurrence of œstrous in spayed females. Removal of the testis eliminates the hormone source and there is no evidence that any appreciable quantity is retained within the organism. This is especially emphasized when one realizes that a ten day hormone output by a normal testicle expresses itself with an increase of ten days in the length of sperm life (as indicated by their motility); similar additional periods can in general be detected by the reaction.

When the aspermatogenetic or cryptorchid testicle is studied it is indeed interesting to learn that a testicle reduced in weight to 0.095 grams produces as much hormone as two normal testicles carrying on spermatogenetic activity and weighing approximately 3.4 grams. These cryptorchid testes had originally produced sperm, but due to experimental elevation into the abdomen their germinal epithelium had undergone dissolution and removal. Sections show the typical picture of natural cryptorchid testes in that the seminiferous tubule outlines are reduced to small cavities with the characteristic single-celled layer of Sertoli elements; between the tubules the interstitial cell masses present the typical picture of "apparent interstitial cell hypertrophy." Such anatomical characteristics have been sufficient stimulus for some writers to designate such an animal as a "supermale" but I have never been able to see adequate justification for the introduction of such a term.

The fact that such cryptorchid testes, having a fraction of the weight of the testicular mass in normal male animals, generated the same quantity of hormone, as do two normal testes (judging from the spermatozoön motility test) suggests again speculation as to the actual elements producing the hormone.

It must be admitted that of all possible elements within the testis, the Leydig cells appear to have the weight of evidence in their favor as being the source of origin of the internal secretions. However, since no one has ever satisfactorily eliminated all other elements such as the general connective tissue, but more especially the cells of Sertoli, there remains the same debatable conditions regarding the actual source of origin. When one views the structure of such degenerate testes as these six and seven month cryptorchid testes (cryptorchid four and five months before epididymal isolation and two months of the experiment) and realizes that each is functioning in producing a full hormone quotient (again judging by the test employed) one is certainly inclined to favor the Leydig cells as the source of origin and to minimize the apparently inactive cells of Sertoli. The question however is not yet settled despite the suggestiveness associated with the Leydig cells.

The chief interest connected with the transplanted testis materials was to see if the spermatozoön motility test would reveal the liberation of any hormone during the process of autolysis of the tissue. Since certain writers had reported such imaginary effects from testis transplantation, it was thought possible that during the breakdown of the incorporated tissues some action might be detected. The results of my investigations, however, have failed to show the liberation of sufficient hormone to be detectable. Despite the capability of the test to reveal hormone action for a period of ten days by a normal testicle it becomes evident that should any hormone effect be derived from transplantation of two entire guinea pig testicles, its effect must be less than that of the normal ten-day production period.

As a further defense of the capability of the spermatozoön motility test to indicate the presence of substances produced by the testicle I may mention that subcutaneous injections of lipoid extracts of the bull testicle, prepared by McGee, and injected by me into guinea pigs whose testes had been removed from their epididymides, resulted in prolonging the life of epididymal sperm to the 54th day after operation (Moore and McGee, '28). Therefore, had the transplanted testis masses been liberating hormone into the host organism, we should have been able to detect it by the test under discussion. Such a result certainly lends no credence to the idea expressed by others that similar non-viable testis grafts in man are sufficiently effective to be noticeable for a period of approximately two years, or again that such transplantations are able to aid in the cure of asthma, tuberculosis, myopia, or the host of other debilities attributed to its effect.

The evidence is very direct that as soon as the hormone producing tissues are removed from the organism the lack of the substance produced begins to be detectable in a very short time. No storage within the body for future utilization is evident.

Summary and Conclusion.

The spermatozoön motility test has been found capable of detecting the production of testis hormone for a period as short as ten days.

The hormone produced by the testicles is not stored within the animal body.

An experimental cryptorchid testicle of five months duration, having a weight of approximately one-tenth of a gram, produces as much hormone (indicated by the spermatozoön-motility test) as do two normal testicles at the height of their spermatogenetic activity. The experimental cryptorchid testis is by weight approximately 2.8 to 3.5 per cent. that of the normal testicular mass.

Autoplastic subcutaneous transplantation of two testes results in the liberation of hormone in such small amounts (if at all) as to be scarcely detectable: if any hormone is liberated by these non-viable testis transplants, the effect upon the animal is no greater than the effect of a ten day normal hormone production. So far as present means will indicate, there is no storage or cumulative effect of the hormone.

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INITIATION OF DEVELOPMENT IN *ARBACIA*. V

THE EFFECT OF SLOWLY EVAPORATING SEA-WATER AND ITS SIGNIFICANCE FOR THE THEORY OF AUTO-PARTHENOGENESIS.*

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In 1901 Hunter published results of experiments which showed that uninseminated eggs of *Arbacia* exposed to sea-water concentrated by evaporation develop on return to normal sea-water. The present writer has been able to confirm these results though his method differs from Hunter's. The sole reason for reporting the findings here presented is that they lead to some interpretations of significance for Lillie's fertilizin theory of fertilization. The work was done during several summers spent at the Marine Biological Laboratory, Woods Hole, Mass.

THE EXPERIMENTS.

Normal uninseminated eggs of *Arbacia*—free of perivisceral fluid, of high fertilizin content, and capable of giving one hundred per cent. fertilization and cleavage—show a small per cent. of cleavage and of abnormal blastulæ that do not rise to the surface, if after having lain in a small volume of normal sea-water for one or more hours, they are removed to a larger volume of normal sea-water. Two conditions are important for this method of initiating development in the egg of *Arbacia*. First, it is best to use fairly dense egg suspensions. The less dense suspensions prolong the time of exposure necessary to initiate development. Secondly, *it is indispensable for the experiment that the dish containing the eggs be left uncovered to insure evaporation.* A concentration of 1 cc. of "dry" eggs plus 99 cc. of normal sea-water was the least dense suspension successfully used. In some cases it was necessary to leave such a suspension uncovered for twenty-four hours before transfer to the larger volume of sea-water; but it was clear here that evaporation was responsible since suspensions of this kind always gave better results when placed in larger dishes with greater surface for evaporation. And if, moreover, a 1 cc. suspension of eggs in 100 cc. of sea-water be poured on a glass plate thereby insuring greater evaporation, the results were indeed striking. However, I am here interested mainly in the results obtained with smaller volumes of eggs and of sea-water.

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In all the experiments it was first ascertained that the eggs to be used were in optimum fertilizable condition by trial inseminations for the estimation of their capacity to separate normal membranes. First, the eggs were carefully collected uncontaminated by perivisceral fluid, washed in four changes of 200 cc. of sea-water, and allowed to settle. The supernatant sea-water was decanted and a highly concentrated bulk of eggs thus obtained. For each experiment the eggs were from one female. These were divided into two equal lots whenever their bulk permitted; one lot was placed in an open dish, the other in a glass vial closed with a ground glass stopper. At intervals of 30 minutes a drop of eggs from each lot was removed to 250 cc. of normal sea-water and their development observed. The per cent. of cleavage was as carefully counted as possible, though the count is often made difficult because of the number of cytolyzed eggs. With further development complete cytolysis of eggs makes the counting of "swimmers" more difficult and of doubtful value since these counts cannot take into consideration eggs that have disappeared through complete disintegration. Moreover, many of the eggs exposed to evaporation develop with their blastomeres separated because the eggs do not possess membranes. In such cases, two "micro-blastulæ" counted may have developed from one egg or each from a different egg, its fellow mass of blastomeres having disappeared completely through disintegration. I therefore early abandoned attempts at making accurate counts and merely noted the presence or absence of "swimmers."

I wish to emphasize that in not one single experiment did I ever find an egg in the stoppered vial that showed the least sign of development. That these eggs were not impaired I determined by inseminating them—in normal sea-water in the case of highly concentrated suspensions, or in the vials in the case of the less concentrated suspensions.

One other point before we consider the experiments in detail. The reader appreciates the fact that the rate of evaporation varied from day to day. I made no attempt to control this variation. It is also obvious that the rate of evaporation depends upon the volume of solution employed—smaller volumes evaporating more rapidly than larger. Finally, the vessels used make a difference; in my experiments I used either shallow dishes, with a large surface for evaporation, or for the greatest volumes of solutions employed glass plates, 30 x 30 cm. For volumes up to 4 cc. Syracuse watch glasses served admirably.

There now follow a summary (Table I.) of the first type of experiment and a brief comment for the purpose of elucidation.

Table I.

The Effect of Slowly Evaporating Sea-water on the Uninseminated Eggs of *Arbacia* as Shown by the Per Cent. of their Development on Return to Normal Sea-water. Experiments on Eggs of 45 Females

No.	Bulk of Concentrated Eggs (in cc.).	Volume of Sea-water (in cc.).	Per Cent. of Cleavage.						Per Cent. of "Swimmers."
			Exp. No.	1	2	3	4	5	Exp. No. 1.
1	0.1	0.9		8	11	7	14	20	9
2	0.5	0.5		7	0	11	14	3	5
3	0.6	1.2		6	12	4	9	0	4
4	1	1		0	6	9	7	2	0
5	1	1		10	7	8	4	14	13
6	1.5	3		21	17	23	27	18	23
7	2	2		0	3	4	0	5	0
8	2.5	5		13	6	18	21	19	15
9	3	3		14	24	5	0	6	10

Eggs from the same females in stoppered vials: No cleavage, no "swimmers" Same volumes of eggs and sea water in each case except as follows: No. 7, 0.5 cc. of eggs + 0.5 cc. sea-water; No. 8, 0.5 cc. of eggs + 0.5 cc. of sea-water; No. 9, 0.5 cc. of eggs + 1 cc. of sea-water.

The data given in Table I. are for eggs exposed to slowly evaporating sea-water for two hours. This one length of exposure is arbitrarily taken for the purpose of simplicity, instead of presenting the results of each 30-minute exposure. In some

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instances the per cent. of development was greater after a longer or a shorter exposure; the results of the two-hour exposure is very nearly the average of all exposures made. Though the per cent. of development in no experiment is high, yet it shows that the evaporating sea-water does initiate development. I do not regard this as an efficient method for experimental parthenogenesis: it has been very suggestive, however, for other lines of my work.

Eggs in sea-water protected against evaporation never show indication that development is initiated. This statement is certainly superfluous for suspensions of uninseminated eggs of *Arbacia* that are ordinarily employed as controls, as all workers know. Of the more dense egg suspensions it might be that lack of oxygen or CO_2 concentration makes initiation of development impossible. The fact that such eggs from such suspension fertilize on return to larger volumes of sea-water does not meet this possible objection. However, I might repeat that some suspensions made of 1 cc. of eggs plus 99 cc. of sea-water exposed to slowly evaporating sea-water showed initiation of development whereas similar suspensions in stoppered vials never did.

Eggs that show initiation of development as the result of exposure to evaporating sea-water never separate membranes, their cleavage is irregular, and the blastomeres tend to fall apart. Many eggs do not cleave and of these some reach the monaster stage with rhythmical dissolution and re-formation of the nucleus. All uncleaved eggs on insemination separate membranes, cleave, and reach the pluteus stage.

The abnormal swimming forms developing from these eggs subjected to treatment with slowly evaporating sea-water never swim at the surface, but merely rotate on the bottom of the dishes; among them are micro- and mega-"blastulæ"—*i.e.*, swimming forms developed from blastomeres that have fallen apart and those developed from two or more cleaving eggs. It is this fact that makes difficult the counting of swimmers; hence, the reader will note that except for the first experiment (Table I.) and for one experiment described below (Table II.), I give no counts, but simply note with a + or o sign their presence or absence.

I interpret these experiments to mean that these eggs in evaporating sea-water are by such evaporation exposed to hypertonic sea-water. It is the hypertonicity that is responsible for the initiation of development and not the mere crowding of the eggs since equivalent volumes of eggs from the same females and of sea-water protected against evaporation do not give any evidence of initiation of development after transfer to larger volumes of sea-water. These eggs as noted above had been thoroughly washed before exposure to evaporation; they would nevertheless continue to produce fertilizin—but so would the eggs protected against evaporation. If fertilizin production, therefore, were responsible for the initiation of development we might expect that at least the highly concentrated eggs in stoppered vials would show some signs of cleavage and farther development. And, what is more, the use of "egg water" instead of normal sea-water does not increase the per cent. or improve the development. Table II. gives the results of a typical experiment on eggs exposed to evaporating "egg water." Drops of eggs from both the uncovered and the stoppered lots were returned at half hour intervals to 200 cc. of normal sea-water. The percentages given are those of eggs having had a two-hour exposure to the evaporating "egg water." This experiment was made five times.

Table II.

The Effect of Slowly Evaporating Egg Water on the Uninseminated Eggs of *Arbacia* as Shown by the Per Cent. of their Development on Return to Normal Sea-water. Experiments on the Eggs of 9 Females.

No.	Bulk of Concentrated Eggs (in cc.).	Volume of Egg Water (in cc.).	Per Cent. of Cleavage.	Per Cent. of "Swimmers."
1	0.5	1.5	9	7
2	0.5	2.5	13	11
3	1	1	7	0
4	1	1	18	14
5	1	2	11	5
6	2	1	3	5
7	2	2	15	12
8	2	3	12	10
9	2.5	5	9	6

Equivalent volumes of eggs from the same females and of "egg-water," except for No. 9 where 0.5 cc. of eggs and 1 cc. of "egg water" were used, in stoppered vials gave no trace of development after return to normal sea-water.

It would appear from a study of Table II. that there is no advantage in substituting "egg water" for sea-water. As a matter of fact, other experiments with "egg water" gave inferior results. In addition, one gains the impression that exposure to evaporating "egg water" causes more eggs to separate blastomeres, and that there are more micro- and mega-"blastulæ." This I did not properly investigate, *i.e.*, by running experiments on lots of concentrated eggs from the same females, one lot exposed to evaporating sea-water, one to stoppered sea-water, one to evaporating "egg water," and one to stoppered "egg water" counting both the eggs that showed blastomeres falling apart and the micro-"blastulæ." However, some older unpublished observations made independently by Lillie and by the writer may be cited. These showed that "egg water" actually possesses a deleterious effect on development. Thus, I found that if eggs from the same female be divided into two lots, one suspended in sea-water and the other in strong "egg water" before or after insemination, the development of the eggs in "egg water" are markedly inferior to that of the eggs in normal sea-water as measured by the per cent. and normality both of cleavage and of plutei. Lillie also has commented on the adverse effect of "egg water" in other ways on eggs. There is indeed no reason why this should not be true and several reasons why it should. "Egg water" is not simply sea-water charged with fertilizin—it contains products of metabolism of the uninseminated eggs, even though metabolism is at a low level; this would be especially true of eggs highly concentrated in strong "egg water," which perhaps also contains more bacteria than normal sea-water.

Glaser likewise notes that "addition of the extracts ["egg water"] in certain concentrations to normally fertilized eggs, resulted in a retardation of development; normal blastulæ instantly slowed their movements, and underwent a noticeable increase in volume when subjected to the extracts. Similar observations were made on the larvæ of *Arenicola* whose rate of movement was also slowed down, to be followed instantly by an outflow of their yellow pigment and a slight reversible agglutination." Unfortunately, however, Glaser's method of preparing his egg extracts—by removing the eggs directly from the ovaries into twice their volume of sea-water—is open to objection since he must have carried over some perivisceral fluid. The perivisceral fluid alone may have been responsible for his results.

The following experiment was also made ten times: eggs from one female were placed (1) in sea-water exposed to evaporation, (2) in sea-water in a stoppered vial, (3) in "egg water" exposed to evaporation and (4) in "egg water" in a stoppered vial; at 30 minute intervals drops of eggs were removed from each of the four lots to dishes each containing 200 cc. of normal sea-water. I give now the summary of one long experiment because it shows the results with varying concentration of eggs from one female:

No. 1. 10 drops of eggs + 90 drops of uncovered sea-water gave 18 per cent. cleavage, + + "swimmers."

No. 2. 10 drops of eggs + 90 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 3. 10 drops of eggs + 90 drops of uncovered egg water gave 6 per cent. cleavage, + "swimmers."

No. 4. 10 drops of eggs + 90 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 5. 20 drops of eggs + 80 drops of uncovered sea-water gave 27 per cent. cleavage, + + "swimmers."

No. 6. 20 drops of eggs + 80 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 7. 20 drops of eggs + 80 drops of uncovered egg-water gave 8 per cent. cleavage, + "swimmers."

No. 8. 20 drops of eggs + 80 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 9. 30 drops of eggs + 70 drops of uncovered sea-water gave 31 per cent. cleavage, + + "swimmers."

No. 10. 30 drops of eggs + 70 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 11. 30 drops of eggs + 70 drops of uncovered egg water gave 11 per cent. cleavage, + "swimmers."

No. 12. 30 drops of eggs + 70 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 13. 40 drops of eggs + 60 drops of uncovered sea-water gave 21 per cent. cleavage, + + "swimmers."

No. 14. 40 drops of eggs + 60 drops of sea-water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

No. 15. 40 drops of eggs + 60 drops of uncovered egg water gave 17 per cent. cleavage, 0 "swimmers."

No. 16. 40 drops of eggs + 60 drops of egg water in a stoppered vial gave 0 per cent. cleavage, 0 "swimmers."

In this experiment because of the rapidity of evaporation on this particular day the eggs were removed to normal sea-water after one hour. The experiment reveals that the effect of evaporating "egg water" is certainly not superior to that of evaporating sea-water in causing initiation of development. It shows also as other experiments cited show that more concentrated suspensions do not yield markedly higher percentages of development than less concentrated ones.

On the whole I think that the evidence which I have submitted indicates that eggs exposed in uncovered dishes develop because of an increasing hypertonicity due to evaporation. Further, the evidence indicates that "egg water" is not necessary for this effect; indeed, "egg water" appears to be less efficacious if not actually more harmful in some small degree than normal sea-water. If this evidence be accepted, Glaser's work on auto-parthenogenesis must be questioned. A brief discussion of Glaser's work and its significance for the fertilizin theory in the light of the work which I herein report now follows.

Discussion.

In 1914 Glaser reported for eggs of *Arbacia* and *Asterias* a type of initiation of development due to exposure to "egg water" for which he chose the name, auto-parthenogenesis. Glaser's procedure was as follows: "Standard secretion ["egg water"] was prepared by adding to a certain number of "dry" ripe ovarian eggs, double their volume of sea-water. At the end of ten minutes, during which the eggs were slightly agitated at intervals, the suspension was centrifuged, and the eggs cast down. After 100 revolutions the supernatant fluid was carefully decanted and set aside for use.

"Ripe eggs were then shaken, usually from the ovaries of a single individual, into a small quantity of fresh sea-water, and to 1 cc. of a concentrated suspension of these was added 1 cc. of the secretion. In this mixture the eggs were allowed to stand 2 hours, when cleavages were usually found in all the dishes."

And further: "Many experiments were tried varying the concentration of the secretion as well as the time of exposure. My records indicate cleavages at higher concentrations as well as lower, and also in less than two hours, but the greatest number was always obtained when 1 volume of the concentrated egg suspension was exposed for 2 hours to 1 volume of the standard secretion. If at the end of this time the supernatant fluid is poured off and replaced by fresh sea-water, free swimming blastulæ will be found within 24 hours. In one case only did development proceed to the pluteus stage."

As I have stated above, Lillie was never able to repeat this observation made by Glaser. Nor was I until by chance I observed the extent of evaporation that had taken place in two cc. of egg water put in a Syracuse watch glass one hour before. Deliberately repeating this chance observation on eggs suspended in either "egg water" or sea-water through several seasons I have obtained initiation of development in *Arbacia* eggs provided the "egg water" or sea-water be allowed to evaporate. I am therefore constrained to believe that Glaser's auto-parthenogenesis is a hypertonic effect due to evaporation.

Glaser has also reported what he calls an improved method of auto-parthenogenesis. Says Glaser: "Loeb's improved method of artificial parthenogenesis consists in following the treatment with parthenogenetic agents, by an after treatment with hypertonic sea-water, 8 cc. of 2.5 M NaCl plus 50 cc. of sea-water. It seemed likely, therefore, that a better yield of larvæ could be secured if eggs, after having been subjected to the action of the secretion for two hours, were afterwards treated with the hypertonic solution for forty minutes. This surmise proved correct." The proof offered is the outline of a typical experiment showing the development in two sets of eggs *both* of which were exposed to hypertonic sea-water after treatment with the egg secretion. There are, it seems to me, two objections to this experiment.

In the first place, in the improved method of artificial parthenogenesis Loeb typically used butyric acid which alone is not capable of causing development of the egg beyond the separation of the vitelline membrane and formation of a monaster around the egg nucleus; according to Glaser, the egg secretion which he used causes development at least to the blastula stage without separation of membranes. Moreover, when one uses butyric acid one must replace the acid sea-water with normal sea-water and allow a certain time to elapse before beginning the treatment with the hypertonic sea-water; Glaser exposed his eggs to the egg secretion and at once transferred them to the hypertonic sea-water. There is here, therefore, no similarity between the improved method of Loeb and that of Glaser.

Secondly, and this is far more serious, Glaser does not tell us to what extent there is an improvement through the after treatment with the hypertonic sea-water; he gives no information concerning the development of two lots of eggs from the same female, one with and one without hypertonic sea-water after the exposure to the egg sea-water. Obviously, Glaser should have set up an experiment on four lots of eggs from the same female, one an uninseminated control in normal sea-water, one exposed to hypertonic sea-water alone, one to egg water alone, and one to hypertonic solution after a treatment with "egg water."

In the same communication Glaser also described auto-parthenogenesis in eggs of *Asterias*. For this he used either 1 or 2 volumes of maturing *Asterias* eggs plus one of "egg water" and obtained fertilization membranes, cleavage, and "much gastrulation." I would suggest that this result was due in part to CO_2, which in *Asterias* eggs initiates development, and to hypertonicity.

Glaser's "hetero-parthenogenesis" is the effect of *Arbacia* "egg water" on *Asterias* eggs. Here again he used 1 volume of "egg water" (from *Arbacia* eggs) to 1 volume of *Asterias* eggs. The foreign "egg water" gave fertilization membranes and numerous cleavages. I venture the opinion that the initiation of development was due to one, two or a combination of three factors: CO_2, hypertonicity, and the foreign perivisceral fluid which from Glaser's method of procuring the *Arbacia* "egg water" must have been present.

On the basis of my findings and the possibility that these adverse criticisms of Glaser's work be correct, I suggest that auto-parthenogenesis is an initiation of development due to hypertonicity of either "egg water" or sea-water. If this be true Glaser's criticisms of Lillie's fertilizin theory based on his findings are without foundation.

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INTRACELLULAR HYDRION CONCENTRATION STUDIES.

I. The Relation of the Environment to the pH of Protoplasm and of its Inclusion Bodies.

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Recent micrurgical investigations (1, 2, 3) on the colorimetric determination of the protoplasmic pH have emphasized the need of studying the relation between the pH of the protoplasm of a living cell and that of its environment. Of the acids and bases which affect the pH of the environment some penetrate living cells while others apparently do not. This has been demonstrated by the change in color of cells stained with indicators. For example, with the use of neutral red it has been shown by previous investigators (4, 5) that living cells are readily permeable to CO_2 and NH_3 but not to HCl nor NaOH. This fact that the color of the intracellular stain can be readily shifted to the acid or the alkaline side suggests that the intraprotoplasmic pH can be changed very easily by environmental conditions, a conclusion which is at variance with experiments which indicate that protoplasm has a marked buffering power. Thus, when solutions of indicators, both in the acid and the alkaline states of their color ranges, are injected into living cells the colors quickly shift to those characteristic of a constant pH (6.9 ± 0.1). This has been found true for such varied types of cells as the ameba (1, 6), marine ova (2, 3), and various tissue cells of the frog and the mammal (6). In addition, there is the significant result that the localized increase in intraprotoplasmic acidity, caused by mechanical injury is almost immediately neutralized as long as no cytolysis results (1, 2, 3, 6).

In view of these facts it was considered advisable to test further the constancy of the intraprotoplasmic pH, to discover whether this pH can be shifted appreciably without detriment to the cell and to obtain evidence, if any, of localized variations in the intracellular pH.

The purpose of the experiments described in this paper is to determine whether the intraprotoplasmic pH can be shifted by exposure to CO_2 or to NH_3 and whether the reaction to indicators of such intracellular structures as granules and vacuoles are comparable to those of the optically homogeneous protoplasmic matrix.

Before dealing with the actual experiments performed it is necessary to describe the manner in which the protoplasm becomes colored with neutral red and with the other dyes used. When cells are stained with neutral red or certain other basic dyes, the dye accumulates in or on the intracellular granules and vacuoles while the hyaline protoplasmic matrix remains colorless. This occurs not only when cells are stained by immersion in a solution of the dye but also when the dye is injected directly into the cell. In the latter case the color appears at first diffuse but gradually the granules and vacuoles take up more and more of the color until none of it can be detected in the hyaline cytoplasmic matrix. On the other hand the acid dyes used, *e.g.*, brom cresol purple, phenol red and cresol red, do not penetrate from the environment into the cells. When injected, however, they quickly spread through the cytoplasm giving to its hyaline matrix a more or less permanent and diffuse coloration (1, 2, 3, 6).

The fresh water *Amœba dubia* and the unfertilized eggs of the starfish, *Asterias forbesii*, and sanddollar, *Echinarachnius parma*, were used in these experiments. The amœba and the eggs were colored with the dyes either by the immersion method or by the microinjection method. Both methods were also used simultaneously on the same cell. The cells were then immersed in various acid and alkaline solutions and the color changes noted. For a study of the effect of NH_3 and CO_2 the cells were suspended in hanging drops of water from the roof of a special form of moist chamber which was closed except for narrow inlet and outlet tubes (7). The hanging drops were then charged with either CO_2 or with NH_3 by passing the moist gas through the chamber.

1. EFFECT OF ACIDS AND BASES ON AMEBÆ COLORED BY THE INJECTION OF ACID INDICATORS ONLY.

Amebæ were injected with 0.4 per cent solution of brom cresol purple, phenol red and cresol red (8). These indicators were selected because they change color within the pH ranges tested (1, 3). Amebæ, injected with brom cresol purple, are uniformly blue (the alkaline range), with phenol red, a pale orange yellow (approaching the acid range). These findings accord with those already published (6) from which the pH of the freshwater ameba was placed at 6.9 ± 0.1.

Amebæ, colored by the injection of the above-mentioned dyes, were immersed in solutions of HCl (pH 5.5), NH_4Cl (pH 5.5), CO_2 charged water (pH 5.5), $NaHCO_3$ (pH 8), NH_4OH (pH 8) and NaOH (pH 8). The acidity of the first three solutions is sufficient to cause the indicators to take on the yellow color of their acid ranges, while the alkalinity of the last three solutions is sufficient to give to brom cresol purple the purple blue, and to phenol red and cresol red the bright red color of their alkaline ranges. It was found that the immersed amebæ all maintained their original colors as long as they remained alive. The color of those which rounded up and died changed to that characteristic for the pH of the environing medium.

These results indicate, either that there is no penetration of the acid or of the alkali from the solutions used, or that the protoplasm is sufficiently buffered to neutralize the acid or the alkali which does penetrate.

2. EFFECT OF ACIDS AND BASES ON CELLS STAINED WITH NEUTRAL RED AND INJECTED WITH ACID INDICATORS.

a. Amœba dubia.

Since the permeability of cells to certain acids and bases can be demonstrated by the change in color of neutral red, amebæ were immersed in a solution of neutral red until various intracellular inclusions took on a red color. These amebæ were then injected with solutions of the indicators which color the cytoplasm diffusely. On immersing these doubly colored amebæ into the various acid and alkaline solutions the following results were obtained:

In accordance with the previous experiment it was found that immersion produced no change whatever in the diffuse coloration of the hyaline cytoplasmic matrix. On the other hand, the inclusion bodies which were stained with neutral red quickly became yellow in the solutions containing the NH_3 (NH_4OH and NH_4Cl) and bright red in those containing CO_2 ($NaHCO_3$ and CO_2 charged water).

These results imply that the pH of the hyaline cytoplasm does not change even when sufficient NH_3 or CO_2 penetrates to change the color of the intracellular inclusions. In other words, the pH of the intracellular inclusions can be shifted readily by the presence of CO_2 or of NH_3 in the environment while that of the protoplasmic matrix remains constant.

b. Unfertilized Eggs of the Sanddollar (Echinarachnius parma) and the Starfish (Asterias forbesii).

The protoplasm of these eggs is uniformly crowded with granules or macrosomes practically all of which ultimately stain a deep rose red with neutral red. The eggs were allowed to remain in sea-water containing neutral red only long enough to stain a small percentage of the granules. The eggs were then washed, transferred to hanging drops of sea-water in the moist chamber and injected with the indicator solutions. In the same chamber were placed, as controls, other hanging drops of sea-water colored with the same indicators. Ammonia gas was then passed through the chamber until the hanging drops became sufficiently saturated with ammonia to change the color of the control drops.

The color of the eggs was noted when the dyes in the control drops had assumed colors indicating a pH more alkaline than 8.4. In every case the color of the granules, stained with neutral red, changed from red (acid) to yellow (alkaline) while the diffuse coloration of the indicators in the hyaloplasm of the eggs persisted in registering the originally recorded pH of 6.8 ± 0.1 (3).

An experiment giving striking color contrasts is one in which three dyes, neutral red, phenol red and cresol red, were used for the purpose of detecting simultaneously the pH changes in the cytoplasm, the cytoplasmic granules, and the sea-water surrounding the eggs. It is to be remembered that neutral red which stains the granules is red at a pH more acid than 6.8 and yellow at a pH more alkaline than 7.4. Phenol red which colors the hyaloplasm is yellow at a pH more acid than 6.8 and red at a pH more alkaline than 7.4, and cresol red which was used for the environing sea-water is yellow at a pH more acid than 7.8 and red at a pH more alkaline than 8.0. The experiment was the following: Eggs, stained with neutral red, were immersed in a hanging drop of sea-water colored with cresol red and were then injected with phenol red. The result was a striking picture of yellow eggs containing scattered red granules and surrounded by a medium of yellow sea-water. Ammonia gas was then passed through the chamber until the cresol red in the sea-water changed from yellow (acid) to red (alkaline). As soon as this occurred the cytoplasmic granules, stained with the neutral red turned yellow (alkaline) while the hyaloplasm maintained the original yellow (acid) color of the phenol red. The result was now a picture of uniformly yellow eggs standing out against a background of red sea-water. Carbonic acid gas was then passed through the chamber until it displaced the NH_3 in the hanging drops. As a result the original colors returned, viz., the sea-water again became yellow, the cytoplasmic granules turned from yellow to red but the cytoplasm itself remained yellow.

Since the cytoplasm has a pH of 6.8 ± 0.1 (3) which is in the acid range of phenol red the above experiment is not suited for detecting a possible effect of the CO_2 on the cytoplasmic pH. For this purpose it is necessary to use brom cresol purple (yellow at a pH more acid than 6.0 and blue at a pH more alkaline than 6.2) which, upon injection, colors the hyaloplasm blue. These eggs were immersed in a hanging drop of sea-water colored blue with the same dye. The hanging drop was suspended in the hermetic chamber through which moist CO_2 gas was made to stream until the sea-water became charged with CO_2 sufficiently to change its color from blue to yellow. The eggs in the yellow water kept their original blue color.

These experiments indicate that NH_3 and CO_2, both of which penetrate the protoplasm and affect the pH of the intracellular granules, do not shift the pH of the hyaloplasm as measured by the indicators.

3. EFFECT OF CO_2 AND OF NH_3 ON AMEBÆ WHOSE CYTOPLASM AND INCLUSION BODIES ARE COLORED WITH THE SAME INDICATOR.

A possible error in the previous experiments lies in the fact that the coloration of the cytoplasmic inclusions and of the hyaline cytoplasm were not made with the same dye. For example, neutral red, which colors the cellular inclusions, is a basic dye, while the dyes used for producing a diffuse coloration are acidic. It is conceivable that this may be responsible for their difference in reaction to the penetrating CO_2 or NH_3.

To meet this objection it was found that methyl red could be used. Methyl red has already been used as a vital stain for plant protoplasm (9) and is a pH indicator, being red at a pH more acid than 5.0 and yellow at a pH more alkaline than 5.4. Immersion of amebæ in an aqueous solution of this dye stains the hyaline cytoplasm, its various inclusions and the nucleus an intense yellow. Amebæ colored in this way were placed in a moist chamber in hanging drops of the yellow aqueous solution of methyl red. Moist CO_2 gas was then passed through the chamber until the hanging drops turned from yellow to red. When this occurred it was found that the yellow stained inclusions of the ameba had also become red while the cytoplasm and nucleus remained yellow. Ammonia vapor was now passed through the chamber whereupon the color of the hanging drops and of the intracellular inclusions quickly changed back to yellow.

These experiments with methyl red clearly demonstrate the penetration of CO_2 into the living ameba[1] as registered by the change in color of the intracellular inclusions. The hyaline cytoplasm and the nucleus, however, maintain their original color and give no evidence of a change in pH.

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[1] The neutral red method is not very favorable for detecting the penetration of CO_2 into cells since the granules stained with neutral red under normal conditions already have the rose red color characteristic for the acid range of the dye. On the other hand, methyl red under normal conditions stains the intracellular granules the yellow color of its alkaline range. Upon exposure to CO_2 the color of the granules changes to red, which is as decided an evidence for the penetration of the CO_2 as is the neutral red method for the penetration of NH_3.

4. The Effect of Penetrating Acids and Bases on the Nuclear pH.

The nuclei of immature starfish eggs were used in these experiments. The nuclei of different eggs were colored with cresol red, neutral red and phenol red by the microinjection method after which the eggs were exposed to CO_2 and to NH_3. In every case the color within the nuclei of living eggs remained constant irrespective of the color changes of the granules in the surrounding cytoplasm. In other words, the nucleus was found to be sufficiently buffered so that the intranuclear pH of 7.6–7.8 (3) remains unchanged. When the egg disintegrates by crushing or tearing, the nucleus undergoes changes (3) and loses all buffering action. The persisting spherical nuclear remnant is then immediately susceptible to acid and alkali changes in its environment.

Summary.

The presence of CO_2 or of NH_3 in the aqueous medium surrounding living cells readily changes the pH of the intracellular inclusions which stain with neutral red but does not change the pH of the protoplasmic matrix nor of the nucleus as long as the cell is alive.

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INTRACELLULAR HYDRION CONCENTRATION STUDIES.

II. The Effect of Injection of Acids and Salts on the Cytoplasmic pH of *Amœba dubia*.[1]

PAUL REZNIKOFF AND HERBERT POLLACK.[2]

In a previous communication (1) from this laboratory the pH of the cytoplasm of *Amœba dubia* was reported to have a value of 6.9 ± 0.1. To determine whether any permanent variations in the intracellular pH could be artificially produced, solutions of acids salts and simple salts having toxic actions were injected by the micrurgical technique (2) into amebæ previously colored with indicators.

Experimental.

The ameba and methods used in these experiments were the same as those described previously (2). The hydrion indicators (3) employed were thymol blue, brom phenol blue, brom cresol green, methyl red, chlor phenol red, brom cresol purple, phenol red, and orange III. Of these phenol red was the most extensively used. The advantages of this dye are twofold. It is the least toxic of all the indicators and is the most valuable one in experiments of this type since its useful range covers the normal cytoplasmic pH. The other indicators were used in extreme changes of pH.

In the case of each solution, at least 10 amebæ were used, and for critical concentrations from 25 to 50. Small quartz cover slips were employed in these experiments. They were attached by means of water films to the ordinary long glass cover slips. On the quartz slips were placed amebæ in a drop of their medium, varying in reaction from pH 5.8 to 7.5, a drop of indicator, and

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[1] From the Laboratory of Cellular Biology, Department of Anatomy, Cornell University Medical College, New York City, and the Marine Biological Laboratory, Woods Hole, Massachusetts.

[2] Expenses connected with this investigation were in part defrayed from a grant by the Ella Sachs Plotz Foundation.

a drop of the solution the effect of which was to be tested. The pipettes used were made of pyrex glass and were rinsed several times in distilled water and then in solutions of the substances to be injected. The dyes were injected into the amebæ which were permitted to recover. After recovery the next solutions were introduced.

As a control, the degree of injury caused by the insertion of the pipette was determined. The method employed was to note any change in color of the previously injected dye from the possible formation of acid associated with injury (4). The simple introduction of a pipette into an ameba was found to give no indication of acid production. If the pipette, however, stirred up the cytoplasm so vigorously that the injured area was ultimately discarded, a distinct acidity was produced. When death occurred in the presence of those dyes covering the range, a pH of about 5.5 was indicated, unless the pellicle surrounding the mass was broken in which case the color was rapidly washed out.

Solutions of HCl (pH 2) when introduced into an ameba, which is colored an orange-yellow with previously injected phenol red, cause an immediate and intense yellow coloration of the injected area. If the injected region is not irreparably injured by the acid the pH of the area reverts within a few seconds to that of the normal cytoplasm. Usually, however, the injected portion is injured to such an extent that it is pinched off in a manner previously described (2) after which the yellow color (acid) of the discarded sphere gradually changes to that indicative of the pH of the environment. In time the color entirely washes out. The unaffected remnant of the ameba retains its orange-yellow color.

When $CaCl_2$ is injected in concentrations stronger than $M/200$ the phenomenon of solidification and pinching off is accompanied by distinct evidence of acid production. If a solution of $M/200$ $CaCl_2$ is injected into amebæ colored with phenol red, the flash of yellow color indicating acid production rapidly returns to that of the normal pH, provided the injected area is not discarded. If the color does not revert within a few seconds the affected portion is pinched off.

In only three cases out of several hundred did the maintenance of a localized acid reaction persist for as long as a minute after HCl or $CaCl_2$ had been introduced without subsequent pinching off. To investigate further this condition in which a localized acid reaction is maintained for an appreciable time with subsequent complete recovery solutions of *M*/32 $AlCl_3$ were introduced into amebæ colored with phenol red. Such a concentration of $AlCl_3$ causes a solidification of the injected portion but this region is not infrequently reincorporated after being almost discarded (5). Of at least 50 cases only one showed a delay of two to three minutes in the return of color from yellow (acid) to the original orange-yellow after the affected area had been reincorporated. In every other test the reversal of color was immediate if reincorporation occurred or, if the area was discarded, its color remained yellow.

The introduction of solutions of $MgCl_2$ of pH 6.5 in concentrations of *M*/30 and stronger into amebæ previously injected with phenol red causes an immediate shift to yellow, indicating acid production. The cell breaks and the color diffuses out. When an *M*/60 solution of $MgCl_2$ is injected the yellow color reverts rapidly to that normal for healthy cytoplasm and the ameba recovers.

In order to determine the degree of acid production by the injection of $CaCl_2$ and MgCl (pH 6.6) amebæ were injected with this salt after having been colored with thymol blue, orange III, methyl red, brom phenol blue and brom cresol green. All these dyes were injected with the exception of methyl red (6). Amebæ were stained with methyl red by immersing them into 5 cc. of distilled water into which a few drops of a 0.4 per cent. aqueous solution of methyl red were placed. With methyl red a distinct red is produced when either $CaCl_2$ or $MgCl_2$ is injected into amebæ. With orange III the yellow color persists. This places the reaction of the acidified portion of the cell between pH 4.0 and 4.6. It is difficult to determine a more exact pH value because the color changes with brom phenol blue are not sufficiently distinctive within the critical range. These results show that the acid produced by injection of $CaCl_2$ or $MgCl_2$ is more marked than the acid of injury which was found to be about pH 5.5.

When NaCl and KCl (pH 7) are injected into amebæ colored with phenol red, no immediate change in color occurs. If the concentration of these salts is lethal (2) the rounded amebæ gradually take on the color indicative of the pH of the environment. Injection of non-lethal concentrations of these salts results during the quiescent period in a slight shift in color toward that suggestive of the pH of the surrounding medium whether this be acid or alkaline. But as soon as the ameba recovers the color reverts to the normal orange-yellow.

The change in color of the discarded spheres after HCl and $CaCl_2$ had been injected or of the dead ameba when lethal amounts of NaCl or KCl were introduced is due to the penetration from the environment. It is quite obvious that any uncontrolled changes in the environmental pH would be confusing. Therefore it was necessary to take precautions to obviate this factor. In preliminary experiments, when ordinary cover slips were used the medium increased in alkalinity markedly during its contact with the coverslip. The use of pyrex glass or coverslips coated with balsam or collodion did not prevent this change in hydrogen ion concentration. To maintain a constant pH of the environment a buffer calcium acetate solution [1] of pH 6 was used. In this amebæ were immersed and the various salts and acids injected. In this case the dead spheres and the amebæ killed with NaCl or KCl took on the color representing the reaction of the surrounding medium, viz., pH 6. With quartz cover slips, which do not affect the pH of solutions coming into contact with them, the medium remained constant and the discarded spheres and dead amebæ assumed the reaction of any environing medium into which they were placed. These results show that the injection of the individual chlorides are ineffective in changing the intracellular pH except when toxic concentrations were used.

DISCUSSION.

In the marine egg the production of acid due to injury is much more easily manifested than in the ameba. As previously shown (4) a localized flash of color indicating acid production is apparent in the starfish egg if the needle is introduced abruptly into the interior. This change is not evident in the ameba unless the mechanical trauma is vigorous enough to cause death of the disturbed part. This difference points to a greater susceptibility to injury of the egg or an increased buffering power of the ameba which may in turn be due either to an increase in the amount of buffer present or to a greater ease in diffusion of buffers through the cell. This faster rate of mobilization of buffers in the ameba as compared to the egg is suggested by the constant flow of cytoplasm of the ameba in contrast to the relatively quiescent cytoplasm of the egg.

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[1] We wish to thank Dr. William Perlzweig for the preparation of this buffer solution.

The production of acid when $CaCl_2$ or $MgCl_2$ is introduced into the ameba may be due to the production of insoluble Ca or Mg salts with the liberation of free acid. Aub and Reznikoff (7) have suggested such an explanation for the effect of Pb salts on red blood cells. Ca may also unite with the carbonate and phosphate to form insoluble salts with the production of free acid. This acid formation is evident until some alkali diffuses into the solidified mass and neutralizes the acids present. Such an explanation does not preclude the possibility also of the formation of a Ca or Mg organic compound.

Conclusions.

1. The cytoplasm of the living *Amœba dubia* shows considerable buffering power to pH changes induced by the injection of salts and buffers.

2. If HCl, injected into the ameba, is immediately buffered by the cytoplasm no toxic effect results. If the quantity injected is too great to be buffered, the affected portion of the cell dies and is discarded.

3. $CaCl_2$, $MgCl_2$ and $AlCl_3$, injected into amebæ colored with indicators, give colorimetric evidence of the production of acid greater in amount than can be explained by acid produced by mechanical injury. Unless this color reverts immediately to that indicative of normal cytoplasm, the affected portion is discarded in the case of $CaCl_2$ and $AlCl_3$ and the entire cell dies in the case of $MgCl_2$.

4. Upon death permeability changes occur so that the dead mass of the ameba quickly assumes the hydrogen ion concentration of the environment.

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INTRACELLULAR HYDRION CONCENTRATION STUDIES.

III. THE BUFFER ACTION OF THE CYTOPLASM OF *Amœba dubia* AND ITS USE IN MEASURING THE pH.

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Recent determinations of intracellular pH have been made by noting the color of indicator dyes injected directly into the protoplasm (1, 2, 3, 4, 5). The recorded value was found by comparing the results of injecting a series of overlapping dyes. The color of the dye, whose range was found to include the pH of the cytoplasm, was compared with known standards projected optically into the field of the microscope.

While attempting to determine the buffer action of the cytoplasm it was found that an indirect method could be used to check the results obtained from the direct color comparisons.

It is known that a drop of a solution at a certain pH added to another buffer solution containing an indicator dye will cause a momentary localized change in the color providing the reactions of the two solutions are different. The closer the pH values of the two solutions are to one another, the less marked will be the color change. When they have the same pH there will be no change in color. It is possible to take advantage of this fact in measuring the intraprotoplasmic pH by injecting a series of solutions of known pH into cells colored by the previous injection of indicator dyes. As will be brought out later, this technique is only approximate but serves to check wide deviations from direct tint comparisons.

It has been shown that $M/4$ solutions of mono-sodium phosphate may be injected with no toxic effect (6), and that the potassium ion has about the same toxicity as the sodium ion on injection (7). Hence the Clark buffer solutions (8) whose KH_2PO_4 concentration is $M/20$ should be non-toxic from the point of view of salt concentrations, and any toxic effect must be due to the buffered hydrogen ion concentration.

Amœba proteus and Amœba dubia were used in this study since their pH has been determined by direct tint readings.

The amebæ were injected with brom cresol purple and phenol red which were the indicators whose ranges cover the pH as found by previous work. The colored amebæ were then injected with the phosphate buffers from pH 5.6–8.0 and observations made on the changes in color.

When buffer solutions of 5.6, 5.8, 6.0 were injected into amebæ colored with brom cresol purple, a temporary but distinct yellow flash was produced. Those above 6.2 produced no color change with this indicator which is already blue in the cell. Buffer solutions of pH 6.2 and 6.4, when injected into amebæ colored orange yellow with phenol red, gave temporary yellow flashes. With the same indicator, solutions having a pH of 6.6, 6.8 or 7.0 showed no color change. Those whose pH was 7.2 and above showed reddish flashes in the orange yellow colored cytoplasm.

This shows that the pH of the amœba is not less than 6.6 and not greater than 7.2. This is in accord with the results obtained in this laboratory in previous investigations and not with those obtained by the Needhams (2). They also used the microinjection technique with direct color comparison for reading the pH values. Their value for the cytoplasmic pH of the amœbæ was 7.6, as was Pantin's, who used the neutral red vital staining technique (9).

As for the Needhams' results it must be remembered that they were using a European species and also that they report the amebæ died within five minutes after injection. In the investigation reported in this paper the amebæ were allowed to recover fully after the injection before treatment with the buffer solutions. With a proper injection of phenol red and brom cresol purple, amebæ can be kept alive and apparently normal for at least two days (4). Frequent checks on the color by direct comparison with standard buffers showed no change during that time. The amebæ colored with phenol red maintained the same orange yellow tint (pH 6.9 ± 0.1) as long as they were kept under observation. On the other hand moribund and dead amebæ take on the pH of the environment which is usually alkaline when the ordinary glass coverslips are employed without proper precautions (5). As for the value obtained by Pantin (8) the inefficacy of neutral red staining has been shown (3, 10).

The interesting fact is that regardless of the pH value of the buffer solution injected the return of color of the indicator present to its usual one is quite rapid and constant. If, however, sufficient buffer was put in to change the pH of the cell, the cell died. These facts emphasize two important points relating to intracellular hydrogen ion concentration. One, that the cytoplasm has a considerable buffering power, and two, that when the pH of the cytoplasm is changed, the cell dies.

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THE EFFECTS OF CHANGES IN MEDIUM DURING DIFFERENT PERIODS IN THE LIFE HISTORY OF *UROLEPTUS MOBILIS* AND OTHER PROTOZOA.

LOUISE H. GREGORY.

3. THE EFFECTS OF YEAST EXTRACTS.

The effect of vitamines on the vitality of protozoa has been a subject of but little investigation. In 1917 Calkins and Eddy (1) reported no effect of treating paramecia with pancreatic vitamine extracted with Fuller's earth. In 1918 Lund (2), working with yeast extracts, found that if Paramecia had been starved before being fed with boiled yeast their size and speed of oxidation were increased but not the cell division. In 1919 Chambers (3) reported a slight increase when the animals were fed yeast, especially ground yeast, and in the same year Flather (4) obtained similar results with the unpolished rice. All of these experiments were upon *Paramecium*, which is not a favorable subject for investigation unless pure lines are established and endomixis watched, for a change in the vitality may be due to a reorganization of the protoplasm rather than to a change in the environment. Abderhalden and Kohler in 1919 (5) reported a slight stimulation of *Colpoda cucullus* when treated with yeast extracts but the evidence is not decisive.

Through the courtesy of Professor W. H. Eddy and Dr. Ralph Kerr, of Teachers College, I have had placed at my disposal the following yeast extracts. (1) Alpha bios No. 223 extracted by Professor Eddy in 1924 (6). (2) Beta bios isolated in 1928 by Dr. Kerr (7). (3) Gamma bios a residue substance similar to bios II reported by Lucas and Miller (8) in 1924. These three substances were prepared as indicated in Table I., which has been compiled by Dr. Kerr.

TABLE I.

THE SEPARATION OF YEAST AUTOLYZATE INTO VARIOUS BIOS FACTORS.

I. *Preliminary Fractionation.*

1. Make autolyzed yeast 66 per cent. alcoholic by volume to precipitate proteins. Filter.
2. Filtrate from 1. Add hot saturated baryta so long as an immediate precipitate

forms. Add alcohol as necessary to maintain 66 per cent. strength. Filter. Save ppt. for 8.

3. Filtrate from 2. Contains alpha bios and some gamma bios. Neutralize immediately with sulfuric. Adjust to pH 4.7. Precipitate with iron sol. and discard pptate.
4 Filtrate from 3. Adjust to pH 5.3 Precipitate with iron sol. Filter. Save filtrate for 7. Precipitate contains all the alpha bios.

II. *Isolation of Alpha Bios.*

5 Ppt. from 4. Work up with water and refilter to remove water washings. Dissolve ppt. in 30 per cent. sulfuric. When solution is complete dilute with water and neutralize with baryta. (Fe, SO_4, ions removed as $Fe(OH)_3$ and $BaSO_4$). Filter by suction and discard ppt. With baryta and sulfuric remove quantitatively all Ba, Fe and SO_4 ions.

6. Filtrate from 5. Evaporate to dryness and recrystallize from hot 95 per cent. ethyl alcohol. Purified product has melting point 223° C.

III. *Concentration of Gamma Bios.*

7. Start with filtrate from 4 Evaporate to small volume. Add sulfuric to make 5 per cent. by weight. Filter if necessary and discard ppt. Now add phosphotungstic dissolved in 5 per cent. sulfuric so long as any ppt. forms. Filter and discard filtrates. Ppt. contains gamma bios.

15. Start with phosphotungstates from 7 and 12. Decompose with baryta in the usual way. Filter. Make filtrate decidedly alkaline to litmus. Add alcohol to 80 per cent. by volume. Filter and discard ppt. if any. Free filtrate of Ba and SO_4 quantitatively. Filtrate now contains a product not yet purified but which suggests Miller and Lucas' bios II. We designate it here as gamma bios.

IV. *Isolation of Beta Bios.*

8. Start with ppt. from 2. Wash with alcohol. Then stir washed ppt. repeatedly with water and filtrate by suction so long as the water is colored. Neutralize the filtrates immediately with sulfuric. Refilter and discard pptates and residue.
9. Filtrate from 8. Treat with hot saturated Ag_2SO_4. Filter and discard ppt.
10. Filtrate from 9. Treat with hot saturated acid mercuric sulfate. Filter. Discard ppt.
11. Filtrate from 10. Free from Ag and Hg ions with H_2S. Remove excess SO_4 with baryta. Save clear filtrates. Evaporate at 40° C. to small volume.
12. Filtrate from 11. Make 5 per cent. sulfuric by weight and extract five times with ether of equal volume discarding ether extract. Add phosphotungstic acid in 5 per cent. sulfuric until no further ppt. forms. Filter. Add the ppt. to 7. (See III, 7 above.) Make filtrate slightly alkaline with baryta and refilter. Discard this ppt.
13. Filtrate from 12. Neutralize with sulfuric and evaporate to small volume. Make 80 per cent. alcoholic by volume and again ppt. with baryta. Filter and discard filtrate.

14. Precipitate from 13. Free of Ba with sulfuric. Evaporate to a thick sirup at 40° C. Dehydrate by stirring and grinding with dry acetone to a fine white powder. Filter nearly to dryness on suction filter but leave enough acetone to make a moist mass. Transfer acetone-wet product to vacuum desiccator and here free of acetone by suction. Product is *Beta bios*.

I wish to express my appreciation to Professor Eddy and Dr. Kerr for their interest and helpful suggestions.

The work with the bioses was begun in 1926–27 at the same time when experiments with di-sodium phosphate were being conducted on *Uroleptus mobilis* in order to determine any variations in response according to the age of the protoplasm. Since then *Dallasia* from pure lines of Professor Calkins, *Stylonychia*, and *Pleurotricha* have been used in addition to *Uroleptus mobilis*. Whenever possible the material was taken from pure lines started from an exconjugant or cyst so that the age of the protoplasm was known. In the case of *Stylonychia* conjugation did not occur and the material was obtained from a single individual isolated from the wild culture. The methods used in all experiments were the same as those of earlier papers and as usual the rate of division is considered an indication of the vitality of the protoplasm.

In earlier papers (9, 10), results of experiments have been reported which indicate that the protoplasm of *Uroleptus mobilis* varies in its response to treatment. Beef extracts and di-potassium phosphate cause an increase in the division rate only when the protoplasm is mature while di-sodium phosphate causes an increase in the division rate of cells of all ages but the greatest increase occurs in the mature cells. Experiments with di-sodium phosphate have been continued and will serve as an additional control in the majority of the experiments with the bioses.

I. *Experiments with Alpha Bios.*

Three series of *Uroleptus mobilis* were used in these experiments. Various concentrations of alpha bios were tried and finally a concentration of .05 mg. per cc. was fixed as the best. The bios solution was added to the normal hay flour medium daily and controls were carried on in the normal medium and also parallel experiments were conducted at the same time with a medium containing di-sodium phosphate in the Packard (11) concentration of $M/7000$. The results are shown in Table II.

TABLE II.

EFFECTS OF DI-SODIUM PHOSPHATE AND ALPHA BIOS ON THE DIVISION RATE OF *Uroleptus mobilis*.

Series No.	Age in Gen.	Amount of Variation from Control in Division Rate per Line in 10-day Periods.	
		Di-sodium Phosphate Series.	Alpha Bios Series.
139	19	+ 3.8	—
	64	+ 3.4	—
	125	+ .6	− 3.0
	175	+ .6	− 2.4
	225	+ 4.4	+ 3.0
	240	+ 1.6	− 2.6
	275	+ 3.4	+ 3.0
140	19	+ .4	—
	60	+ 2.4	+ 1.8
	117	− 3.0	− 3.2
	157	− 1.0	− 4.8
	218	+ 3.4	+ 2.0
	227	+ 4.2	+ .6
141	6	+ 1.2	− 1.8
	36	− 1.0	− 2.2
	66	+ 1.8	− 2.2
	125	+ 1.6	− 2.4
	180	+ 4.2	+ 1.9
	192	+ 4.4	+ 2.4
	234	+ 3.2	+ 4.0
	243	− .6	− 4.0

As in former experiments the sodium phosphate caused a stimulation of the vitality throughout the life history of Series 139 and practically throughout the life of Series 141. Series 140 was the least vigorous and died out in the 227th generation after showing instability throughout its life. The greatest stimulation however in all three series appeared during maturity.

Alpha bios failed to act as a stimulant save in the 225th and 275th generation of Series 139, three times at slightly irregular intervals in Series 140 and only slightly after the 180th generation in Series 141 save in the 234th generation when there was a slightly higher division rate than that of the control or sodium series. Thus alpha bios with few exceptions has a depressing effect on the vitality of these three series of *Uroleptus*.

These results may be due to at least two factors: (1) too acid a condition of the medium, (2) a general lowering of the vitality of *Uroleptus mobilis*. Undoubtedly the protoplasm was weakening as it did not respond as vigorously to sodium stimulation as it did in the experiments of 1926. On the other hand the H ion concentration of the alpha bios medium was slightly lower than that of the normal medium (7.2). Since trial experiments with beta bios known to be more acid, resulted in a decided lowering of the vitality and since it seemed uncertain whether any bios would cause a definite stimulation of an animal cell, experiments were conducted in which a yeast extract containing all the bioses was used and in one series di-sodium phosphate was added to the yeast extract medium to increase the alkalinity.

II. *Experiments with Harris Yeast Extract.*

Four experiments with *Uroleptus* at varying ages and two with *Dallasia* were carried on in which the animals were kept (1) in a normal medium to which was added daily yeast extract of a concentration of .01 mg. per cc., (2) in a normal medium to which was added di-sodium phosphate, (3) in the same medium as in (2) with the addition of the yeast extract and (4) in normal hay flour medium as the control series. These results are shown in Table III.

TABLE III.

EFFECTS OF YEAST EXTRACT ON THE DIVISION RATE OF *Uroleptus mobilis* AND *Dallasia*.

Series No.	Age in Gen.	Amount of Variation from Control in Division Rate per Line in 10-day Periods.		
		Sodium Phosphate Series.	Yeast Extract Series.	Sodium Phosphate Yeast Extract Series.
Uroleptus				
146....	15	died	− .2	died
143....	30	− 2.0	− 3.6	+ .4
142....	84	− 3.4	− 2.8	− 2.6
141....	250	0.0	− 5.2	− 2.8
Dallasia				
1......	75	− 3.4	+ 5.0	+ 9.0
2......	75	+ 3.2	+ 7.2	+ 10.4

The experiments with *Uroleptus* show practically no stimulating effect of the yeast extracts. The protoplasm was too weak to respond and the entire race died out shortly afterwards. The two experiments with two different series of *Dallasia* both in the 75th generation, showed a definite increase in division rate in all the experimental series. It was especially marked in the yeast sodium hay-flour medium when the rate was 10.4 divisions higher than that of the normal control series and 12.4 higher than the sodium hay-flour series for the same period. Since the yeast extracts caused a marked stimulation of the vitality of *Dallasia* both with and without the addition of sodium to the normal medium the fractional extracts of the yeast were then used.

III. *Experiments with a Neutral Salt of Beta Bios and with Gamma Bios.*

Dallasia, *Pleurotricha* sp. and *Stylonychia* sp. were treated with the two bioses using the same methods as above. These three animals differ in their normal rate of cell division. *Dallasia* when young undergoes from 3–5 divisions daily, *Pleurotricha* like *Uroleptus* not more than 1–2 divisions and *Stylonychia* divided every other day. *Stylonychia* may have been more mature as in this series no conjugations occurred and the age is unknown. The results of the experiments are shown in Table IV.

TABLE IV.

THE EFFECTS OF BETA AND GAMMA BIOS ON THE DIVISION RATE.

Series No.	Age in Gen.	Amount of Variation from the Control in Division Rate per Line in 10-day Periods.	
		Beta Bios Series.	Gamma Bios Series.
Dallasia 2.........	115	— .8	+ 4.8
	130	+ .2	+ 4.8
Pleurotricha	90	+ 5.0 (1st 10 days) died (2d " ")	+ 10.2 (1st 10 days) + 1.6 (2d " ")
	110	—	+ 4.8
Stylonychia		— —	+ 3.8 (1st 10 days) + 4.8 (2d " ")

Beta bios apparently had no effect on *Dallasia* when older gamma bios, however acted as a definite stimulant increasing the division rate, 4.8 divisions per line in 10 days. *Pleurotricha* was stimulated in the 90th and 110th generation and showed a marked response to gamma bios. *Stylonychia* had its division rate almost doubled in the gamma bios solution and this effect continued for twenty days. When *Pleurotricha* was stimulated for twenty days the effect died out during the second ten-day period. This may be correlated with the variation in normal vitality of the two species.

IV. *Experiments with Alpha, Beta and Gamma Bios.*

Finally experiments were conducted to compare the effects of the three bioses on *Pleurotricha* and *Stylonychia*, when added to the normal medium and in a few experiments to the sodium medium. The results are shown in Table V.

TABLE V.

EFFECTS OF ALPHA, BETA, AND GAMMA BIOS ON THE DIVISION RATE OF *Stylonychia* AND *Pleurotricha*.

Amount of Variation from the Control in the Division Rate per Line in 10-day Periods.						
Alpha Bios Series.	Beta Bios Series.	Gamma Bios Series.	Na_2HPo_4 Series.	Na_2HPo_4 Alpha Bios.	Na_2HPo_4 Beta Bios.	Na_2HPo_4 Gamma Bios.
Stylonychia + 1.4........	+ 4.2	0.0	. + 4.4	—	—	+ 3.2
Pleurotricha 115 gen +10.2	+ 11.4	+ 10.2	+ 8.2	—	—	+ 16.0
130 gen. died..	+ 4.8	+ 8.0	− .8	died	9.0	+ 12.8

In these experiments, *Stylonychia* quickened its division rate in all media save that of the Gamma Bios, where the division rate equalled that of the control. *Pleurotricha* in the 115th generation showed a definite stimulation in all media, especially in that with the HNa_2PO_4 and gamma bios. In the second experiment the division rate of the control dropped to 5.4 divisions per line in 10 days while the experimental series kept a much higher level, the climax being reached with 18.2 divisions per line for the same 10-day period in the series kept in normal medium to which HNa_2PO_4 and Gamma Bios solution had been added.

Summary and Conclusions.

These preliminary experiments indicate that while the protoplasm of *Uroleptus mobilis* was usually depressed when treated with yeast extracts due probably to its weakened condition, that of *Dallasia*, *Pleurotricha* and *Stylonychia* were definitely stimulated by the addition of fractional extracts of yeast to the normal medium. Alpha bios in general causes the least effect and gamma bios the greatest increase in division rate. While there is usually an increase in the protoplasmic activity when sodium phosphate is added to the medium already containing the bios solution, this may not be due to an increased alkalinity as the variation in Hydrogen ion concentration were not more than .1–.2 of a point. The explanation may lie in an increase in the permeability of the cell allowing a far more reaching effect of the bios solution. The age and general characteristics of the protoplasm must also be taken into consideration and further experiments are planned with pure lines of varying ages.

The fact of a sudden marked increase in the division rate of a protozoan cell when treated with these yeast extracts brings to mind the theory of Burrows in which the rapid growth of cells and formation of tumors is assumed to be due to a lack of balance between vitamines in the cells. The relation of these extracts to vitamines has yet to be proved. They are however stimulating substances to yeast cells and to certain animal cells, their effects varying according to the age and general conditions of the protoplasm.

Barnard College,
June, 1928.

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BIOLOGICAL BULLETIN

INSECT METABOLISM.

THE ANAËROBIC METABOLISM OF AN INSECT (ORTHOPTERA).

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That insects can live anaërobically for varying periods of time has been repeatedly pointed out (Winterstein, 1921; Lee, 1924, 1925; Willis, 1925; Davis and Slater, 1926, etc.). When deprived of oxygen they enter into a state closely resembling anesthesia. Various methods for the withdrawal of oxygen have been experimentally employed, such as replacing the air by the gases hydrogen, carbon dioxide, nitrogen, etc.; by evacuating the vessel in which the insects are contained and by immersing the insects in water. The results produced by all of these methods closely resemble each other and if the deprivation of oxygen has not been too long the insects recover and appear quite normal. A state of anaërobiosis thus produced in insects offers rather unique conditions for studying the gaseous exchange of an organism during oxygen lack.

The present work deals with the rates of oxygen consumption and the blood pH changes in grasshoppers under normal as well as anaërobic conditions.

MATERIAL AND METHODS.

The grasshoppers, including individuals of the following species, *Melanoplus differentialis*, *Melanoplus femur-rubrum*, and *Chortophaga viridifasciata*, were hatched and raised under laboratory conditions and fed lettuce. Organisms of known and varied ages were used in the experiments. Oxygen determinations were made by means of the modified Krogh Manometer

(Bodine and Orr, 1925), immersed in a Freas constant temperature water bath maintained at 25° ±0.1° C. Animals were subjected to anaërobic conditions largely by immersion in water at 25° C. for varying periods of time. Immersion in water was found to give results identical with those produced by the gases hydrogen, carbon dioxide or nitrogen.

Animals were first put in the manometers and their normal rates of oxygen consumption determined. After removal from immersion in water they were again quickly put in the same manometer and their rates of oxygen consumption during recovery noted. By such a procedure a continuous record of the oxygen consumption of the organism was obtained except for the actual period of immersion in water.

Blood pH determinations were made by micro-colorimetric methods (Bodine, 1925). By means of fine capillary pipettes blood was easily obtained from minute punctures made by fine needles in the lateral abdominal wall of the animal.

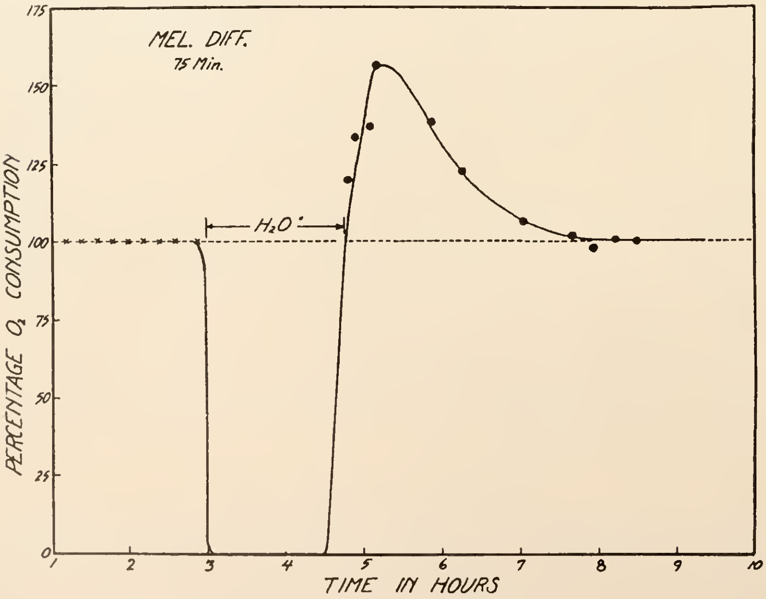

FIG. 1. Curve showing the effect of immersion in water for 75 minutes on the rate of oxygen consumption of a male, nymph, *Melanoplus differentialis*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

RESULTS.

Oxygen Consumption.

The normal rate of oxygen consumption for each organism was determined over a period of an hour or more until a constant rate was obtained. The animal was then removed from the manometer, placed in a glass tube, the open ends of which were covered with wire gauze, and immersed in water at 25° C. to a depth of 180 mm. All air bubbles were removed from the surface of the animal and the ends of the tube by gentle shaking. The animal becomes motionless within a very short time after immersion and remains so throughout the immersion period. After immersion, the organism is quickly removed from the glass tube, dried on filter paper and returned to the same manometer originally used to determine its normal rate of oxygen consumption. The recovering animal is left in the manometer and its rate of oxygen consumption followed until complete recovery

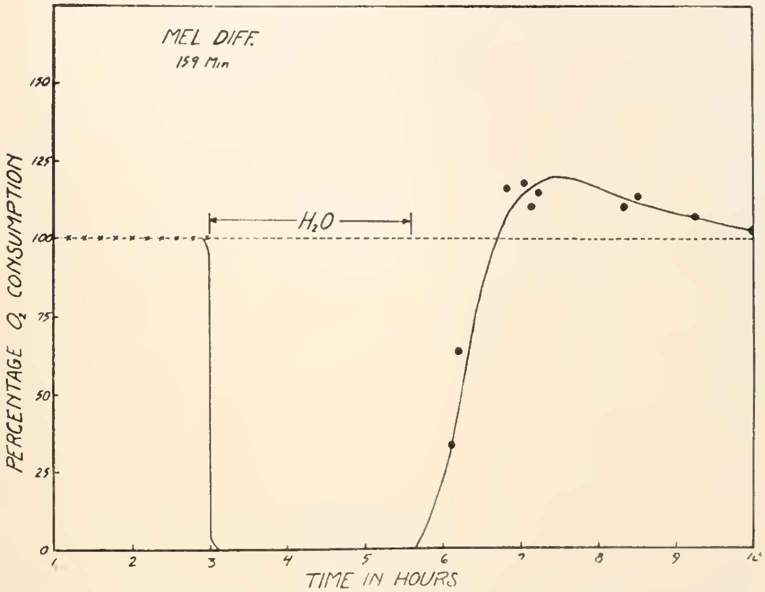

FIG. 2. Curve showing the effect of immersion in water for 159 minutes on the rate of oxygen consumption of a male, nymph, *Melanoplus differentialis.* Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

occurs. Since the general response of all organisms is essentially the same, only typical experiments will be presented.

Figures 1, 2, 3 and 4, in which the rates of oxygen consumption are expressed in terms of the normal rates (100 per cent.), show graphically the changes in the rates of oxygen consumption produced by exposures to lack of oxygen. An examination of these figures further shows that when the animal is readmitted to oxygen after immersion its rate of oxygen consumption increases considerably over the normal rate or that characteristic for the animal before deprived of oxygen. This excess oxygen taken up by the organism can be shown, in carefully controlled experiments, to be approximately equal to the amount the organism would have taken up normally during the period it was deprived of oxygen. In other words, it seems that the grasshopper when deprived of oxygen or existing anaërobically, goes into debt for oxygen in a manner quite similar to that pointed out for the

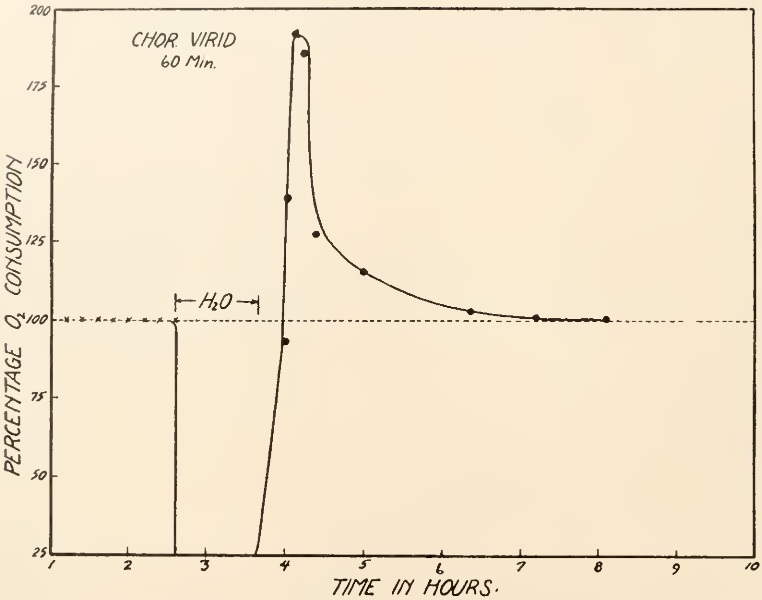

FIG. 3. Curve showing the effect of immersion in water for 60 minutes on the rate of oxygen consumption of a male, numph, *Chortophaga viridifaciata*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

cockroach (Davis and Slater, 1926) and for heavy physical work in man or for isolated muscle (Hill, 1922).

The length of exposure to lack of oxygen that can be withstood by different species of grasshoppers varies as pointed out below. Some species have been found to successfully withstand as high as 7 hours immersion in water. The rates of oxygen consumption during recovery, as indicated in Figs. 1, 2, 3 and 4, seem to be greatly influenced by the length of the immersion period. Animals immersed for 60 to 120 minutes usually recover in a typical manner as shown in these figures. When the immersion period is lengthened, however, there is a strong tendency for the rates of oxygen consumption to return to normal in an extremely slow fashion as shown in Figs. 2 and 4. This slowness in recovery to a normal rate of oxygen consumption is probably correlated with the physiological condition of the organism as well as with the fact that the exposure might be just a sub-lethal one for the animal.

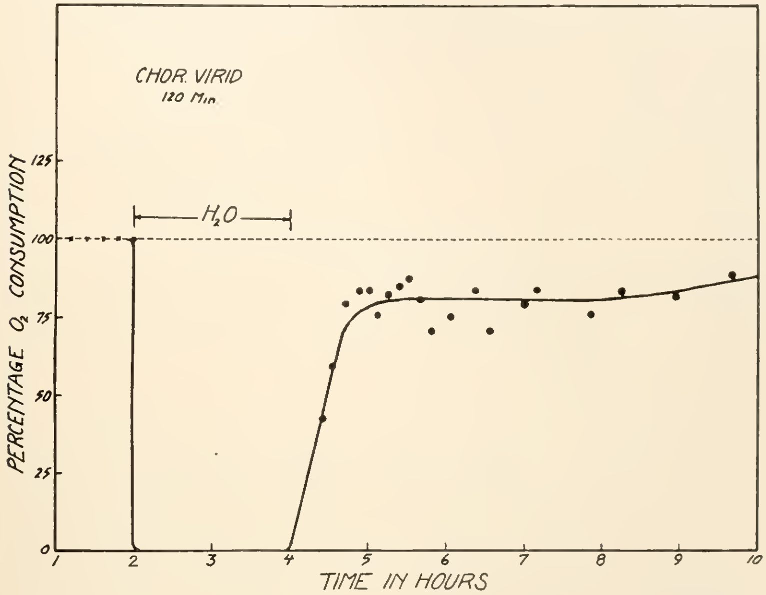

FIG. 4. Curve showing the effect of immersion in water for 120 minutes on the rate of oxygen consumption of a male, nymph, *Chortophaga viridifasciata*. Space within arrows indicates the period of immersion of the animal; points on curve, the rates of oxygen consumption before (which is taken as 100 per cent.) and after immersion. Abscissa, time in hours.

There also appears to be a marked difference in the rates of recovery in the different species. *Melanoplus differentialis* seems better able to reversibly withstand long immersion than *Chortophaga viridifasciata*, as indicated in Figs. 2 and 4. Age is also an important factor, since younger individuals withstand and recover from long immersions better than older ones.

The relations between length of immersion in water and recovery time for the different species of grasshoppers examined are graphically shown in Fig. 5. From a study of this figure it is evident that a linear relationship exists between length of immersion and recovery time. That the causes of the anæsthetic condition produced by lack of oxygen are doubtless due to the carbon dioxide and lactic acid produced within the organism seems reasonable when a comparison is made between the results obtained by immersion in water and those obtained by subjecting the organism to carbon dioxide (Willis, 1925), as indicated in Fig. 6. These curves are qualitatively similar. In view of similar evidence gained from studies on anaërobiosis of mammalian muscle (Hill, 1922), such an hypothesis seems reasonable in explaining these phenomena in grasshoppers. As a matter of

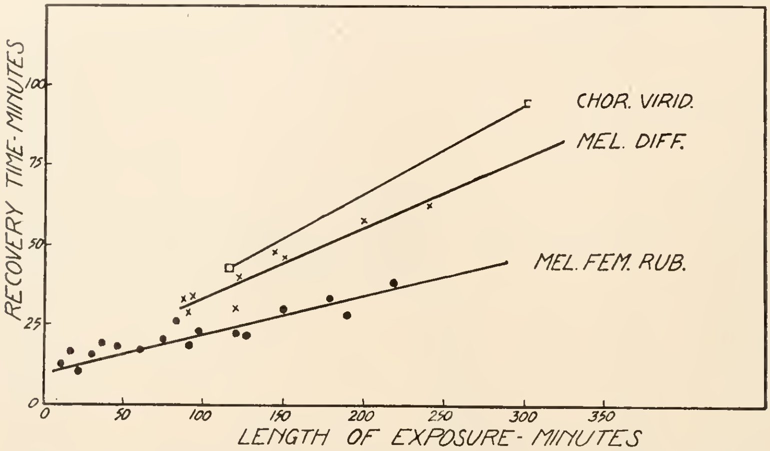

FIG. 5. Curves showing relation between average mean recovery time and length of immersion in water in young adult grasshoppers of three species, *Melanoplus femur rubrum*, *Melanoplus differentialis* and *Chortophaga viridifasciata*. Each curve based on several hundred observations. Recovery time indicates return of "turn-over or righting" reflex.

fact, Lee (1924) has shown that injection of carbonic acid or lactic acid into a grasshopper produces results quite similar to the present ones on immersion of the animals.

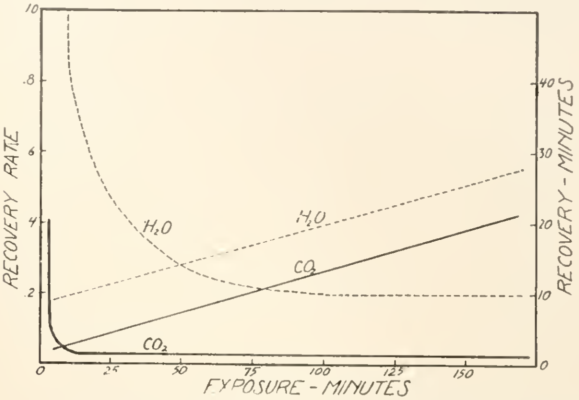

FIG. 6. Curves showing comparisions between average mean recovery times and rates and length of immersion in water and exposure to carbon dioxide in *Melanoplus differentialis*. Carbon dioxide data from Willis (1925).

BLOOD pH.

As previously pointed out the normal pH of the blood of the grasshopper, *M. differentialis*, seems to be 6.8. (Bodine, 1926.) A careful study has been made of the blood of individuals throughout the present immersion and recovery experiments. Fig. 7, in which are plotted together results of experiments carried out for different periods of time, shows graphically the pH changes occurring in the blood during the anaërobic and recovery periods. A progressive lowering in pH values with length of immersion occurs and seems to reach a minimum at about 5.8. Below this minimum the animal seems irreversibly affected. Upon recovery, a slow blowing off of acids (H_2CO_3), probably occurs and the blood then gradually returns to its normal pH value. An interesting fact, indicated in Fig. 7, is that no marked changes in blood pH occur in immersed animals when removed from the water until after respiratory movements have become established. Upon careful examination of the animal it is found that initial respiratory movements upon recovery are extremely slow and of great depth. As recovery progresses the movements become more regular and normal. These changes in blood pH strongly suggest that during anaërobiosis large quantities of acids, carbonic and lactic, are produced and that recovery consists largely in their elimination by the organism.

As pointed out by Davis and Slater (1926), who have found similar results for the anaërobic metabolism of the cockroach, insects seem to be extremely favorable material with which to further elucidate the changes occurring during the anaërobic existence of organisms and the question of energy formation under such conditions.

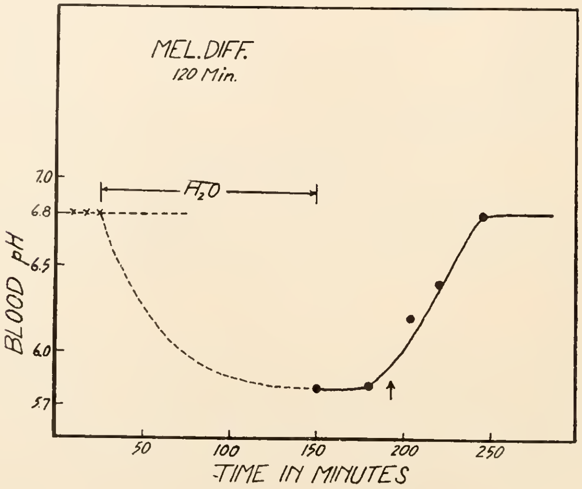

Fig. 7. Curve showing the effect of immersion in water for 120 minutes on the pH of the blood of a male, nymph, *Melanoplus differentialis*. Space within arrows indicates period of immersion of the animal; points on curve, actual pH determinations of blood before and after immersion. Broken portion of curve is composite, being made up from pH determinations of the blood of individuals immersed for periods of time ranging from 50 to 120 minutes.

Summary and Conclusion.

1. Rates of oxygen consumption in grasshoppers before and after immersion in water (lack of oxygen) have been determined.

2. During oxygen lack, grasshoppers build up an oxygen debt. When readmitted to oxygen an increased rate of oxygen consumption occurs and an excess of oxygen, approximately equal in amount to that which the organism would have taken up normally during the period it was deprived of oxygen, is consumed.

3. During anaërobiosis blood pH falls. Upon recovery pH values slowly return to normal.

4. It is suggested that the chemical change responsible for the anesthetic condition accompanying anaërobiosis is the production of an excess of acid, carbonic and lactic, and that recovery consists in their elimination.

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THE PULSATORY RHYTHM OF THE CONTRACTILE VESICLE IN *PARAMECIUM.*

FRANCIS E. LLOYD AND J. BEATTIE.

I.

It became apparent to the senior author a year ago that there are discrepancies between the more recent, and therefore presumably the most correct, accounts of the behavior of the contractile vesicle in *Paramecium* and the objective facts as apprehended by him. As to these facts both the present authors have found themselves in agreement, and it was therefore determined to make an investigation of said behavior by such refined means, by way of control of direct and unaided vision, as are available. Two methods were used, that of recording visual observations of critical points in the contractile cycle on a rotating drum and, still better, that of making motion pictures at normal speed, viz: 16 per second, without any lapse. It is not easy to get a *Paramecium* to remain within the field of vision long enough to take a motion picture so as to get a continuous view of the contractile vesicle for two or three cycles of movement. We have however succeeded by making use of very slight compression between cover slip and slide—a method to which there is some, but we believe not wholly justified objection—and by surrounding the animals with a suspension of Chinese ink, a time honored method for demonstrating the expulsion of fluid from the contractile vesicle, as used by Carter (1861) and by Jennings (1904) and by many others before and since. It happens that a rather thick suspension of this insoluble pigment impedes the movements of the animal, so that the chances for observing a relatively quiet one, with the contractile vesicles in full view, is much increased without, we think, in the least affecting the behavior of the vesicles themselves. We suspect that in Chinese ink there is an admixture of some aromatic substance which may act as a mild depressant, narcotic or otherwise, but of this we have no sure knowledge. However, the animals can live for several hours under a cover-glass in a thick suspension of Chinese ink and appear quite undamaged. We have no doubt at all events that the behavior of the vesicles is normal. The motion pictures produced by the senior author have been shown [1] in illustration of this paper; in the accompanying plate we present a few excerpts from one of the films to serve present purposes.

The point of departure of this study may be better appreciated by considering in the first place the latest pronouncement on the subject, that of Nassonow (1924). In the text which follows we shall speak simply of the vesicle [2] (= contractile vacuole) and the canals (= rays, canaliculi, radial canals). The general topography of the apparatus is so well known that we may take familiarity with it for granted. Nassonow says:

"After the emptying and the complete disappearance of the vesicle there become visible in its immediate vicinity the 5–7 canals. The ends directed toward the center of the vesicle are strongly swollen and no continuity between them is to be seen. The swollen ends approach each other, flow into each other and form a new vesicle, into which the fluid of the canals now flows. Thereupon the canals quite disappear and only later do new canals begin to be formed in their place the ends of which after the emptying and disappearance of the vesicle suffers enlargement and in this manner complete the cycle" (Nassonow, 1924,[3] p. 454). Nassonow then goes on to recall the views of others, including that of Stempell (1914) in regard to the existence of a membrane, with which Nassonow was particularly concerned, apparently acquiescing completely with this author in respect of the progress of the cycle of behavior. We therefore quote Stempell also as follows:

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[1] Winnepeg meeting of the Royal Society of Canada, May, 1928.

[2] It seems to us that Claparede and Lachmann chose the better terminology, and we follow them, with Pritchard.

[3] Among the figures illustrating the paper by Nassonow occur two which we may remark in passing to be capable of precisely the opposite interpretation to that given by him. His Fig. 40 is labelled "Diastole of the excretion apparatus" while Fig. 42 is labelled "Systole of the excretion apparatus." If by excretion apparatus he means the vesicle then figure 40 represents early systole and figure 42 early diastole. If however he means the canals then his labelling is correct. It is not easy to understand his exact meaning. It is certain that diastole and systole of the vesicle are not synchronous with those of the canals.

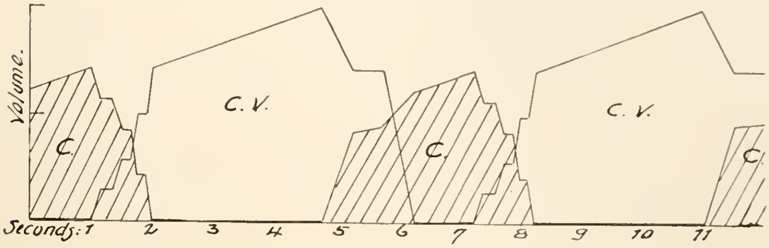
"The end-products of metabolism collect in dissolved condition in certain places in the protoplasm, namely, in a canal- or space system (probably a branched one) the exits of which run towards the two pulsating vesicles as afferent canals. As soon as the vesicle is emptied ('Sobald die Vacuole sich entleert') the ends of these afferent canals swell up to form 'Bildungsvacuole' since the fluid flows hereinto as to the place of minimal pressure, and is here dammed up. As a result of this pressure delicate protoplasmic valves open and permit the volumes of fluid which have collected in the canal-ends to flow together in the vesicular space, on which, after this is filled, the valves promptly close. Since the fluid now collected in the vesicle has a high osmotic pressure, there results a lasting addition of water to the fluid already held in the vesicle by diffusion through the semipermeable membrane formed *ad hoc*. As soon as the pressure of the fluid in the vesicle has reached a certain height, that is, has become higher than that of the external water pressure, a second valve at the apex of the papilla-like dome of the pellicula opens and there results from the pressure of the protoplasm and of the surface tension of the vesicular drop a complete emptying of the vesicle, whereupon the process is repeated in the same way" (Stempell, 1924, p. 460). Here it may be remarked that Fortner (1926*a* and *b*) and v. Gelei (1926) without inquiring into the validity of the above view, proceeded on the assumption of its truth.

With regard to Stempell's ideas, as above expressed, there can be no doubt since he has furnished us with a diagram. From both description and diagram we learn that Stempell does not entertain at all the idea that there is any *flow of fluid from the vesicle into the canals*. Nassonow's idea is identical, we believe we are right in saying, but his diagram, taken from Pütter (1903), might be interpreted otherwise, as witness Figs. 5, 6 and 7, which show the canals enlarging before the contraction of the vesicle. Whether this enlargement results from backflow from the vesicle, or from the collection ("ponding back" as Carter expressed it) of fluid derived from the surrounding protoplasm, is the question with which we are concerned. We are now in a position in the second place to examine the view of earlier observers of the same phenomenon.

Felix Dujardin (1841) made a drawing, reproduced in his Plate

8, Fig. *6a* and *6b* in which the radiating vesicles ("taken by Ehrenberg for seminal vesicles") are seen in the condition just before the systole of the vesicle, the canals being expanded. Dujardin does not, of course, have anything to say about the matter, but his drawing could not be correct if the canals do not fill before the systole of the vesicle. The question above indicated therefore recurs, whence the fluid which fills the canals.

As to this there was no doubt in the minds of Claparède and Lachmann (1858) (Lachmann 1857 for 1856). It was these who held the view that the apparatus is the homologue of the circulatory apparatus in the more differentiated animals and it was consistent with their view that there was no opening affording an exit for the fluid to the outside. Their unfortunate error in this seems to have led to a general condemnation of their whole conception and thereby their critics, in overlooking what they did see, fell into an error as grievous, namely, in failing to see that the canals are in the first instance filled at the expense of the vesicle, as we hold. Lachmann's description (1857) will serve our turn at the moment.



He says (1857, p. 224), maintaining that the thin area of the body wall over the vesicle is only a thin place fit for diffusion and with no opening, that when the vesicle is fully expanded the canals are fine lines. By the sudden contraction of the vesicle, however, the canals instantly swell into pyriform spaces close to the contractile vesicle, which has disappeared. During the slow reappearance of the vesicle, the canals gradually decrease and they have again been reduced to fine lines by the time the vesicle has become fully inflated.

It must be clear that Lachmann believed that the swelling of the canals is synchronous with the early period of systole of the vesicle. Carter (1861) does not, we think, correctly take his meaning when he says: "Claparède and Lachmann have said that the fluid of the vesicle is returned into the vessels on the systole or contraction of the vesicula because the sinuses and vessels become filled *immediately afterward*" (italics ours) as this is not what Lachmann said.[1]

It is in this connection that Carter suggests that the swelling of the canals into the characteristic pyriform is due to the "ponding back" of the fluid which flows through the canals into the vesicle for the short time that the latter empties itself, like the ventricles of the heart but in the other direction (1861, p. 282). We may here remark, what we shall endeavor to show to be true, that the rate of swelling of the canals does not consist with the idea that the fluid reaches the lacunæ by diffusion through the walls, the rhythm of diastole and systole in these being of the same character as in the vesicle; and, if the rhythm of the vesicle can be understood only when it is admitted that the fluid of the canals gushes into it, the same must be admitted for the canals, but in the opposite sense.

Somewhat earlier, and in contrast to Lachmann and Claparède Lieberkühn (1856), while agreeing with them as to time relations of vesicular systole and canalar diastole, saying that "a little before we observe the commencement of the systole, the vessels begin to expand slowly, etc.," simply denied that there is any backflow. The interest here is obviously the correct observation in regard to time relations in question. Spallanzani also believed that the canals become empty as the vesicle fills, and do not reappear until some time after it has contracted and that therefore "The fluid with which the vesicula is distended comes through the sinuses, but is not returned by them to the body" (through Pritchard, 1861).

J. Müller (1856) appears, according to Claparède and Lachmann (1858, p. 51), to have taken the same view of the time relations. We transcribe their summary of his views, since we have been unable yet to see Müller's original paper. This author distinguishes in the behavior of "central circulatory apparatus" of *Paramecium* two partial systoles which alternate with each other—the systole of the vesicle, then the systole of the fusiform or pyriform swellings. The latter coincides with the diastole of the vesicle. Lieberkühn had already observed that "un instant avant le systole des vesicules les rayons se renfluent considerablement." Müller explains the phenomenon by showing that the vesicle contracts, diminishing insensibly in volume in the instant which precedes systole and forces at once a part of its contents into the "rays of the star." Then the systole of the vesicle takes place, which produces a further swelling of these rays.

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[1] We have not been able to see Claparède's paper, but it appears that these two observers, Claparède and Lachmann worked in harmony, sharing each other's views.

We cannot refrain from mentioning, in passing, the work of Wrzesniowski (1869), who studied *Enchelyodon*, *Trachelophyllum* and *Loxophyllum*, (but was however chiefly concerned with the question of the absence or presence of a contractile membrane), because there is some evidence in his results which point to the presence of a contractile vesicular apparatus similar to that of *Paramecium*, though the author himself, if he adhered to the original account, would deny this. One point may be mentioned, however, namely, that a series of small vesicles is formed on the surface of, and from the contractile vesicle during early systole, and these, upon growth, run together later to form a new contractile vesicle (not the old one reëxtended). This view of Wrzesniowski's seems to be strongly linked with his conviction that the vesicle is formed *de novo* and totally lacks a membrane in any but the sense of molecular physics as Khainski (1911) would express it.[1]

We pass to the year 1883 when Maupas attacked the subject. According to him the systolic movement of the vesicle is sudden and rapid. A little before it happens the canals commence to fill in the form of elongated pears at a little distance from the point where they open into the vesicle. Maupas' account indicates a high degree of meticulous care in observation He goes on to remark for example that the systole of the vesicle takes place more often before the pyriform swellings (of the canals) have attained their full size. In spite of the fact that he correctly apprehended the time relations involved he pronounces for the view that the canals are simple afferent conduits and sententiously remarks "I have never seen thé liquid of the vacuole reënter them." This would indeed be difficult and his failure cannot be charged to his discredit, for at all events he very correctly describes the at first irregular contours of the vesicle during the early stages of its diastole when, under systole of the canals, these empty themselves into the vesicle. Maupas was on the side of the non-membranists.

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[1] Samuelson observed in 1857 that the single globular vesicle in *Glaucoma scintillans* when it contracts forces the fluid into others which appear temporarily around it.

It will be seen that these earlier observers, while disagreeing in regard to the afferent-efferent nature of the canals, support a majority view which, as we believe, correctly describes the time relations between the behaviours of the vesicle and the contributory canals. It is therefore a curious fact that later observers, as we have already shown at the outset of the paper, siding with the view of the solely afferent nature of the canals have in some way been led to overlook the true time relations.

Closely connected with the general trend of inquiry above outlined is the parallel inquiry into the nature of the membrane lining the vesicular cavity It will be easily apprehended that very convincing evidence has been so difficult to obtain that only recently has Miss Howland (1924) favored the view that a proper membrane in the morphological sense is present constituting the branching cavity composed of the central vesicle and its contributory canals. She succeeded in isolating the membrane with little distortion by micro-dissection from an animal (*Paramecium caudatum*) treated with a strong solution of alizarin blue. This author expressed some doubt of her interpretation based on the possibility that the dye had coagulated the surface material of the vesicle and so produced an artefact. In the same year Nassonow presented evidence based on the method of osmication which would convince even the elect were it not for a doubt similar to that expressed by the former author. We venture to think that a weak link exists in the chain of his argument. We are not here concerned with this author's views of the homology of the contractile vesicle with the Golgi apparatus although we subscribe to the general view supported by Nassonow that the pulsating vesicle is a true organelle of morphological value, as Lachmann so long ago held. With regard to earlier observers it will boot us little to bring forward the details of their views, a summary of which will be found in a paper by Taylor (1923). Fortner, by compressing animals in a hypertonic solution of cane sugar, was able to set free the apparatus surrounded by protoplasm and in a state of approximate diastole. Their behavior he argues unqualifiedly postulates the impermeability of the membranes; but these membranes he believes arise *ad hoc*, that of the vesicle at the completion of each systole affording the new membrane for the papilla pulsatoria. Without further discussion of this matter from the historical point of view we may be permitted to remark that had the true time relations in the cycle of events not been lost sight of, the protagonists of the "non-membranous" view would have suffered pause.

II.

No special technique is required to demonstrate the phasic activity of the contractile vacuole and canaliculi in *Paramecium*. Care must be taken that the cover slip over the preparation does not press untowardly on the animal, otherwise the pore to the exterior may be blocked and the contractile vesicle fail to discharge in the normal manner, and at normal rate.

After the preparation has been made it is well to allow some minutes to elapse before the preparation is examined as it is a hopeless task to attempt to observe the contractile vesicle in one single animal, while the animals are in rapid motion immediately after they have been placed on the microscopic slide. In a short time the animals settle down to feed, and it is then possible to watch a whole group and to pick out one animal for observation. It is possible also to trap the animals in a very fine capillary tube and so limit their movements except round a longitudinal axis. A better method, but open to the objection of an abnormal environment, is to mix finely ground China ink with the mounting medium. This appears to impede the movements and so far as one can see there is no interference with the normal cycle of events within the contractile vacuole system.

After close observation for a few minutes the following series of changes can be seen The contractile vesicle will be observed as a highly refractile almost spherical droplet lying in the most superficial part of the cytoplasm. When the animal rolls over on its side it will appear that at one point there is a close attachment of the vacuole to the pellicle. At this time the vacuole when viewed from the side will appear as three quarters of a sphere with a conical apex attached to the pellicle. When the animal rolls so that the vacuole is observed from above with careful focusing a bright minute ring will be seen in the center of a small clear area in the pellicle. This is the pore through which the vesicle expels fluid to the exterior. The vesicle gradually enlarges and in doing so changes its shape from the conico-spherical form to a perfect sphere. Enlargement after the spherical shape has been attained is slow and very small in amount as to linear dimensions. Suddenly at the end of diastole the vesicle appears to get smaller (Plate I, Figs. 3 to 4) and *at the same moment, not afterwards,* radiating canals appear surrounding the vacuole (Plate I, Fig. 3). Seen from above the inner ends of these structures are separated from the vacuole by a distinct area of protoplasm. Seen from the side the bulbous or pear-shaped ends of the canals are observed to lie in the most superficial layer of cytoplasm and to be continued more distally into the deeper parts of the cytoplasm as fine canals.

After this phase, which can only be interpreted as a distinct diastole of the canaliculi caused by systole of the vacuole and not merely as a damming back of liquid attempting to flow into the vesicle, the vacuole suddenly contracts (Plate I, between Figs. 4 and 5) and expels the remaining contents to the exterior. Therefore systole of the contractile vesicle consists of two distinct phases:

(*a*) First, an early systolic phase during which the contractile movement of the vacuole is slow and diastole of the canals rapid (Plate I, Fig. 3, 4).

(*b*) Second, a later period during which the vacuole expels the remainder of its contents to the exterior (Plate I, Fig. 5).

The behavior as thus set forth has been displayed graphically in the accompanying diagram, in which, to some extent provisionally, we have attempted to express the time relations seen in the rhythm of the contractions and expansions of vesicle and canals, while the volume relations are avowedly inexact, but approximate. Time is plotted on the abscissa, and the volume of the canals and of the vesicle on the ordinates, the total volume of the vesicle being taken as one. The hatched areas are bounded by the curve of diastole and systole of the canals; the areas bounded by the curves for the vesicle are left blank.

There is no doubt that there is a discharge of vesicular contents to the exterior. Jennings showed this first convincingly as has been stated above and we have been able to confirm his observations and to make a motion picture of the process.

There has been some doubt expressed as to whether or not the pore through which the vacuole discharges can admit fluid from the surrounding medium. We have found no evidence to support this theory. All our observations go to show that after the vesicle has discharged its contents reconstitution of the vacuolar space takes place by the discharge into the collapsed cavity of the fluid contained in the canals (Plate I, Figs. 1–2; 6–8). Discharge of the contents of the canals into the vesicular space takes place within one second after the completion of systole of the vacuole. The canals do not however discharge simultaneously but by careful observation one is able to make out that first one canal may discharge into the collapsed vesicular region which then forms an irregular angular cavity [1] soon followed by another and then by the remainder. When the last canal has discharged the space is seen to be conico-spherical as described above. It is possible to analyse the discharge of the canaliculi into the vacuolar space only by study of the motion picture film.

After the reconstitution of the vesicle enlargement takes place and this phase of diastole of the vacuole occupies the longest period of the cycle of events. One notices that the conico-spherical form persists for quite a time (almost three quarters of diastole) before the spherical form is assumed. Once the vesicle becomes spherical systole of the structure takes place within a second or two.

The cycle of events occupies normally about eight seconds. Records which we have made show that in fresh specimens cycles of seven and one fifth seconds were common. We have observed cycles which required ten seconds for completion. When the cycle lengthens it is the diastolic period which is chiefly prolonged. When the animal is compressed gently it is possible to occlude the pore and so prevent the second phase of systole taking place. The first phase, *i.e.* diastole of the canals takes place but there is no discharge to the exterior. In a short time the canals reappear and so the cycle goes on. The vacuole continues to enlarge and before very many minutes the pellicle ruptures and the protoplasm is extruded carrying with it in some cases the entire contractile vesicle. The vacuole may be seen lying as a spherical body in the surrounding fluid. We have not observed any sign of a canal when the protoplasm is examined after bursting.

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[1] Beautifully recorded by Nassonow, Fig. 42.

When neutral red is used in solutions of one part to four hundred or higher concentrations it is frequent to observe the gradual contraction of the cytoplasm from the pellicle and the formation of a peri-cytoplasmic space filled with fluid. In one specimen of which a photograph is shown (Plate I, See Fig. 9) the cytoplasm in contracting pulled a fine cone of pellicle downwards. At the apex of this was attached the pulled-out contractile vesicle which extended as a conical cavity through the peri-cytoplasmic space to the dimple in the pellicle. The actual interface between the vesicle and the surrounding fluid could be seen. The apex of the dimple was the pore through which the vacuole discharges. It also would appear to show that the vacuole when it discharges to the exterior is not reconstituted *de novo* in the old site but rather that there is something of a permanent nature—a vesicular membrane into which is discharged the contents of the canaliculi when the vesicle is reformed.

Miss Howland, as we have already said, has been able to isolate the vesicular membrane from preparations treated with alizarin blue.

By way of summary of the above we draw attention to the following important facts.

Diastole of the vesicle falls into two phases—an early rapid and a later slow one.

The early rapid phase is due to the systole of the canals during which their fluid content is forced into the vesicle.

The later slow phase of diastole of the vesicle is due to further distention by diffusion of water into the vesicle.

Systole of the vesicle falls similarly into two phases, an early slow phase during which the fluid is forced into the canals (diastole of these) and a later rapid phase during which the remainder of the vesicular fluid is forced through the spore into the surrounding medium.

It appears that early diastole of the vesicle is synchronous with the systole of the canals; and that early systole of the vesicle is synchronous with diastole of the canals. During early diastole of the vesicle, this is partly filled with fluid from the canals, This is the residual fluid plus that which has in the interim entered by diffusion into them. During early systole of the vesicle the canals are partly filled with fluid from it—this we may speak of as the residual volume. That volume which is discharged by the vesicle is the overplus accumulated by diffusion into the vesicle and canals during their diastolic periods.

Viewed thus, the mechanism is one in which a certain quantity of fluid of relatively high osmotic pressure is retained in the canals, derived by them from the central vesicle, and which is at once put into service to withdraw water from the body into the pulsatory apparatus. Thus an important feature of Stempell's view receives support, even though his conception of the methods of working of the apparatus is incomplete. If it depended solely upon diffusion for filling, from the completely collapsed state to the completely replete, it could, in our opinion not work so rapidly and efficiently.

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EXPLANATION OF PLATE.

FIGS. 1–8. Eight episodes from motion picture (photomicrographic) of *Paramecium caudatum.* The animal was slightly compressed between slip and cover; the periodicity was slightly slower then normal therefor. The position in the film is indicated for each. Exposure 16 per second. Enlargements at constant distance.

FIG. 1. Foot 887 frame 16. Mid-diastole. Canals are emptying into vesicle.

FIG. 2. 883–1. Late diastole. Traces of canals visible.

FIG. 3. 880–16. Early systole of vesicle; canals beginning to fill.

FIG. 4. 876–10. Mid systole of vesicle which is now smaller; canals nearly filled.

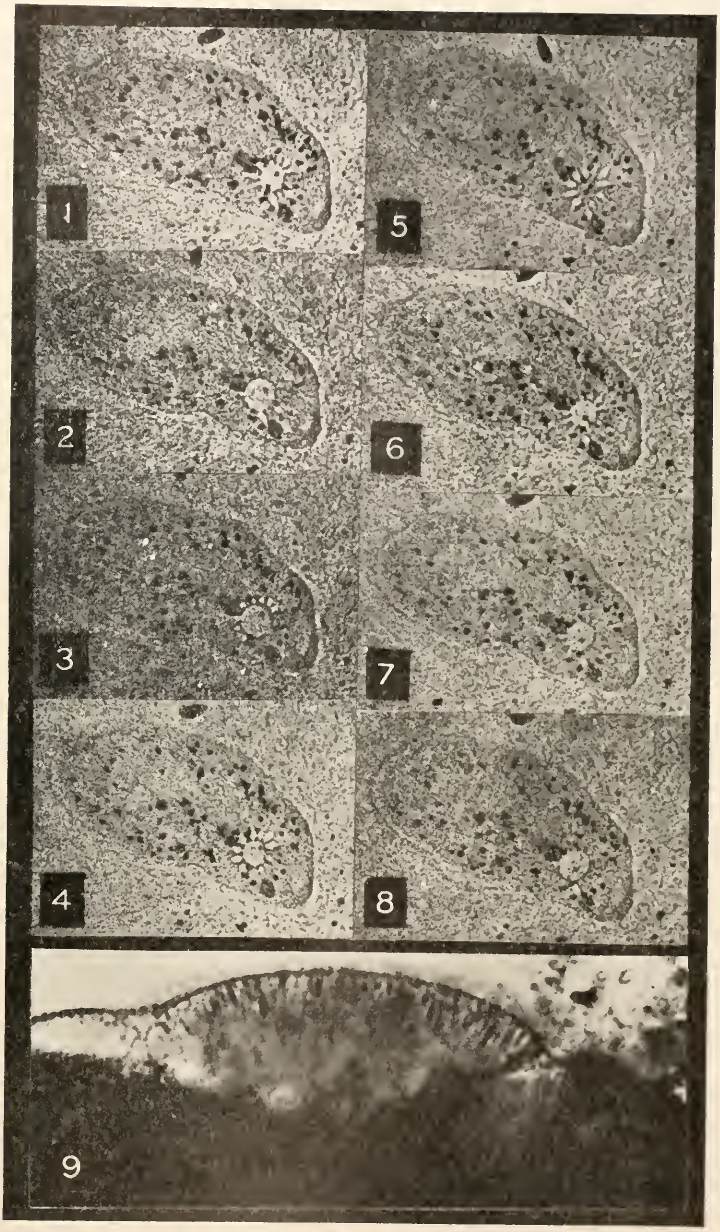
FIG. 5. 873–15. Systole of vesicle complete, canals full.

FIG. 6. 871–10. Mid-diastole (somewhat later than Fig. 1); canals emptying into vesicle.

FIG. 7. 870–3. Later diastole; canals nearly disappeared.

FIG. 8. 868–9. Diastole complete; canals empty. One canal persists longer than the others: note that it occurs in Figs. 1, 2, 5–8.

FIG. 9. An animal treated with neutral red (see text), showing the vesicle pulled away from the pellicle, and dimpling it by pulling on the pore rim. The result follows from the shrinkage of the cytoplasm.



FRANCIS E. LLOYD AND J. BEATTIE.

OBSERVATIONS ON *HYDRA* AND *PELMATOHYDRA* UNDER DETERMINED HYDROGEN ION CONCENTRATION.

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Much has been written recently concerning reduction, dedifferentiation and resorption in *Hydra*. It is generally conceded that reduction in hydra is accompanied by a loss of tentacles. The literature enumerates the following causes by which hydras lose their tentacles. N. Annandale ('07) observed, in studying *Hydra orientalis*, that during the hot season of the year this species has but four tentacles while during the cold season it has six tentacles. G. Entz ('12) observed that an infection with *Amœba hydroxena* may lead to a degeneration of tentacles. Reynolds and Looper ('28) have come to the conclusion that this parasite is responsible for the degeneration of the tentacles. Certain ciliates recorded by E. Reukauf ('12) and P. Shultze ('13) also caused the loss of tentacles. E. Shultz ('06) observed that hunger set up a process of dedifferentiation within the tentacles. Huxley and DeBeer ('23) observed that adverse environmental conditions accelerate dedifferentiation and resorption of the tentacles of *Obelia* and *Campanularia*. They also found that this process of dedifferentiation and resorption might involve not only the tentacles but also part of the zoöid. Berninger ('10) found that, in response to inanition, hydra lost its tentacles. Finally Kepner and Jester ('27) also observed that the loss of tentacles was brought about in response to inanition. This loss, according to them, was accomplished by ingestion of the tips of the tentacles through the mouth. This may occur, but undoubtedly is not the usual method, as Hyman ('28) indicated.

It is a well known fact that the concentration of the hydrogen ion medium that bathes the protoplasm or protoplasmic tissue has a profound effect upon it, therefore it seems strange that no attempts have been made to account for reduction, dedifferentiation and resorption on the basis of such environmental conditions. The following observations and results have been obtained through an effort to determine whether or not the concentration of the hydrogen ion is an important factor with reference to the three above mentioned phenomena.

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[1] These investigations were carried on under the direction of Professor W. A. Kepner. Acknowledgments are due Mr. Carl H. McConnell of this laboratory, for the preparations of the photomicrographs.

Methods and Materials.

Filtered spring water in 300 cc. portions kept in thoroughly cleansed glass dishes was used as a culture medium.

Very dilute solutions of *N*/20 sodium hydroxide and of hydrochloric acid were used in quantities to adjust the pH of the solutions. The colorimeter method was used for the pH determination of the solutions and LaMotte color standards were employed for color matching. Tests, adjustments and observations were made every twenty-four hours except where otherwise indicated. The temperature was maintained between 18 and 22° C. During these investigations frequent examinations were made of both the culture and of the animals for protozoa which might have been responsible for reduction. None were found except where stated. Observations were made with a dissecting binocular of a magnification of twenty diameters. These observations were supplemented by histological preparations.

At first distilled water was tried as a culture medium with the idea that a more accurate determination could be made of the hydrogen ion concentration. Various deleterious factors enter into the use of such a medium so it was discarded. In the subsequent experiments, filtered spring water was used.

The terms reduction, dedifferentiation and resorption, as used by other authors and us, may be defined as follows: Reduction is a uniform decrease in surface area in which process the ectoderm, mesoglea and endoderm remain intact and maintain a normal position in relation to each other. Dedifferentiation and resorption represent a dual phenomenon which involves a local reduction of surface. The presence of this dual phenomenon in the tentacles is indicated by a thickening and knobbed appearance at the tips of the tentacles.

Experimental.

Culture 1.—Four *Pelmatohydra oligactis* (Pallas), were placed in a culture medium consisting of distilled water and NaOH was added to maintain a constant pH of 7.8. At the end of a period of six days there was much apparent reduction and resorption of the tentacles in all specimens. One polyp was fed on the sixth day and one on the seventh. At this point the experiment was terminated through an accident.

Culture 2.—Four *Pelmatohydra oligactis* were placed in a culture medium of distilled water. The culture maintained a pH of 6.8 without the addition of either hydrogen or hydroxyl ions. These polyps disintegrated in five days.

Culture 3.—Four *Pelmatohydra oligactis* were placed in a culture medium consisting of distilled water. This culture maintained a pH of 7.0 which was fatal to the polyps in five days. At this phase of our observations we came to the conclusion that we were imposing other factors than the controlled pH represented, upon the hydras in using distilled water. A change in osmotic pressure was undoubtedly involved when distilled water was used instead of spring water. So, from this point on, spring water was employed as the medium in which to keep the observed polyps.

Culture 4.—Four *Chlorohydra viridissima* (Pallas) were taken from spring water which tested pH 7.6. They were normal in every respect. The pH of the second lot of spring water was now maintained at 6.6. The only change being made here was using a second glass dish similar to the one in which the pH tested 7.6 and in the pH now being 6.6. In five days, six of the polyps had disintegrated and the remaining one had undergone advanced dedifferentiation and resorption. It was placed in filtered spring water of pH 8.6 in an effort to bring about regeneration but it disintegrated in a few hours. This result, together with general observations made on various cultures, in the laboratory, in which the polyps displayed marked dedifferentiation and resorption, indicates that the acid condition of the medium induces dedifferentiation and resorption. Our observation upon a lower hydrogen ion concentration (higher pH) proved to be little more instructive as seen by the following culture.

Culture 5.—Six *Pelmatogydra oligactis* were isolated in filtered spring water the pH of which was maintained between 7.6 and 8.2. On the 8th day all of the hydras appeared perfectly normal; however, on the 9th day, all except one had disintegrated. The one remaining hydra showed no apparent reduction or dedifferentiation and resorption of the tentacles. This hydra was sectioned and its histology appears later in the paper.

On several occasions similar results were obtained when the pH was held within the range from pH 7.8–8.0. It appears that the first ten days represent a critical period when the polyps are exposed to inanition. After the 10th day has passed we have had uniform results as the following observations indicate.

Culture 6.—Four *Chlorohydra viridissima*, in which some resorption was displayed, were isolated in filtered spring water pH 6.6. This water was over *Elodea* which had been previously boiled. The *Elodea* was separated from the polyps by a double thickness of cheese-cloth spread over the bottom of the container. The *Elodea* was removed after six days and spring water alone was used. As indicated above, these hydras were in a somewhat resorbed condition. The pH of this culture was varied, first decreasing the concentration of the hydrogen ions after the first two days up to 7.6, then increasing to 7.0, then again decreasing to 7.8. A pH of 7.8 was maintained for the last thirteen days. Immediately following these changes in pH, we observed the physiological aspect of the polyps. It was seen that the greater the concentration of the hydrogen ions the greater was the degree of dedifferentiation and resorption in the polyps. If the concentration of the hydrogen ions was lessened the hydras returned to normal. Two of the four hydras survived for a period of twenty-three days. One of these was sectioned (its histology is referred to later in the paper) and the other was lost during a transfer for examination. On the nineteenth day a green hydra, with much resorbed tentacles and bearing gonads, was introduced into this culture. In two days this hydra had gained its normal appearance but its gonads had partially disappeared. It was fed and placed in an aquarium containing food where it developed into a fine vegetative specimen apparently normal. In this last specimen the change from laboratory culture water to filtered spring water must have been a factor as well as the change in pH.

This does not however lessen the significance of the reaction of the other individuals of culture 6, wherein only the pH concentration has been the factor involved.

Culture 7.—Six *Chlorohydra viridissima* in a slightly resorbed condition were placed in filtered spring water without *Elodea* the pH of which tested 8.6. After the first two days the pH was maintained at 7.8 until this experiment was terminated. On the fourteenth day one hydra was sectioned. At the end of a period of twenty-four days three hydras remained. They were much reduced in size but their tentacles were apparently normal. On the twenty fifth day they were placed in an aquarium containing food where they lived for several days and attained nearly normal size. At this point our observations on these animals ceased.

These most interesting cases (cultures 6 and 7), in which the polyps that had been reduced and in which apparent dedifferentiation and resorption had taken place at a hydrogen ion concentration above the optimum, were restored to a completely normal condition when subjected to hydrogen ion concentration at or near the optimum. This undoubtedly indicates that food is not necessary for the regeneration of hydra, but regeneration depends rather upon the hydrogen ion concentration of the culture water. Kepner and Jester ('27) record one hydra which had lost all of its tentacles and without the presence of food the lost tentacles were replaced by regenerated ones in eight days. As the culture medium was frequently changed it is probable that a favorable pH was accidentally maintained. Hyman ('28) records the same phenomena when she says: "Depressed specimens may be caused to regenerate if the water is replaced by culture water" (page 78). Huxley and DeBeer in working with *Obelia* and *Campanularia* were unable to cause the regeneration of dedifferentiated and resorbed tissue.

Culture 8.—Eight *Pelmatohydra oligactis* were isolated in filtered spring water the pH of which was maintained for the first two days at 8.4 and for the remainder of the period it was kept at pH 7.8. On the tenth day three hydras had completely disintegrated without displaying reduction, dedifferentiation and resorption. On the 17th day, *Halteria* appeared in the culture. These were not abundant, about ten being found in the field of the binocular dissecting microscope. As all the hydras appeared in the same condition one was sectioned. These sections showed no *Halteria* present within cœlenteron or the food vacuoles. But menatocysts were present in the epitheliomuscular cells of the endoderm and within the cœlenteron, hence the histology indicates that resorption had taken place. This resorption was so slight that it is overlooked by examination of the living polyps under a dissecting microscope. The culture medium was changed, so as to have water free of protozoa, and the observations continued. On the twenty third day one hydra was sectioned (its histology is referred to later). On the twenty fifth day the remaining three hydras were given bits of liver which they readily accepted. Thus indicating that they were not in a "depressed" condition as described by Hyman ('28). They were placed in an aquarium containing food where they were observed for several days. No indication of "depression" became evident during these observations nor was there any evidence of it at the time the observations ceased.

In order to determine wherein the optimum range of hydrogen ion concentration for the medium lay, both green and brown hydras were exposed to varying degree of hydrogen ion concentration ranging from pH 5.2–8.0 and the time recorded when all hydras had disappeared in each culture. The result of this experiment is given in the following table.

Four more cultures were run, with both green and brown hydras, one with a pH of 7.8, the other at pH 8.0. All the polyps in these cultures were alive at the end of a period of twenty four days.

This indicates that the optimum hydrogen ion concentration lies near pH 7.8. And further hydrogen ion concentration is an important factor in the determination of dedifferentiation and resorption; for, in the same medium (filtered spring water) with only the concentration of hydrogen and hydroxyl ions altered, we have been able to either induce or inhibit dedifferentiation and resorption. This does not support the later part of Hyman ('28) page 93, paragraph 2, Biological Bulletin volume LIV, January 1928, number 1 in her explanation of the phenomenon of depression when she says that "it is induced by transfer to clean fresh water." It is quite evident that, if two different lots of hydra

TABLE I.

THE x MARK INDICATES THE DAY OF THE DEATH OF THE LAST HYDRA IN THE CULTURE.

	Existence in Days.											
	2	3	4	5	6	7	8	9	10	11	12	13
Brown hydra in pH 5.2	×											
Green " " " 5.2		×										
Brown " " " 5.4		×										
Green " " " 5.4			×									
Brown " " " 5.6			×									
Green " " " 5.6			×									
Brown " " " 5.8			×									
Green " " " 5.8			×									
Brown " " " 6.0					×							
Green " " " 6.0					×							
Brown " " " 6.2					×							
Green " " " 6.2					×							
Brown " " " 6.4						×						
Green " " " 6.4						×						
Brown " " " 6.6						×						
Green " " " 6.6						×						
Brown " " " 6.8							×					
Green " " " 6.8							×					
Brown " " " 7.0							×					
Green " " " 7.0							×					
Brown " " " 7.2								×				
Green " " " 7.2							×					
Brown " " " 7.4									×			
Green " " " 7.4							×					
Brown " " " 7.6												×
Green " " " 7.6										×		

taken from the same culture or aquarium are placed in identical spring water cultures save for the concentration of the hydrogen ions and favorable reactions are repeatedly to be noted in the culture of low hydrogen ion concentration while unfavorable reactions are always to be noted in the culture of high hydrogen ion concentration, undoubtedly the pH of the culture must be a strong factor in determining this difference in the reactions

HISTOLOGY.

The histology of reduction, dedifferentiation and resorption in Hydra has been observed by E. Shultz ('06) and W. Rehm ('25). Huxley and DeBeer ('23) also described histologically dedifferentiation and resorption in *Obelia* and *Campanularia*. Our observations are almost in exact agreement with those of the above.

Studies on reduction, dedifferentiation and resorption always

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involve the histology of the animal. Hydra is a diploblastic animal having only an ectoderm and endoderm. The ectoderm presents in its vegetative condition, epithelio-muscular, interstitial, cnidoblastic and nerve cells. The endoderm, on the other hand, is made up of epithelio-muscular, glandular, interstitial and scattered or isolated nerve cells. In the ectoderm there is no great local specialization or differentiation into regions. The ectoderm, however, shows three distinct regions: (1) the oral two-thirds in which there are scattered gland cells and a general covering of epithelio-muscular cells that are heavily charged with absorbed alimentary products; (2) a basal third that has few if any gland cells and in which the epithelio-muscular cells are usually highly vacuolated, except for those at the basal disc and (3) the endoderm of the tentacles. In this third region there are no gland cells and the epithelio-muscular cells are highly vacuolated. Thus it appears that the endoderm of the highly active or moving tentacles resembles that of the relatively quiet basal third of the body proper.

Dedifferentiation and resorption have been referred to frequently above. This has been defined as a dual phenomenon which involves a local reduction of surface. We take the presence of ectodermal elements (nematocysts being the most easily recognized) within the coelenteron or endoderm as evidence that dedifferentiation and resorption have taken place.

The question now remains: How is the surface reduced locally, and how do ectodermal elements gain their entrance into the coelenteron? As this phenomenon is most often seen in the tentacles, we have studied it there. In response to adverse environmental conditions, the cells at the tips of the polyp's tentacles coalesce or become dedifferentiated. The ectoderm is apparently affected first. Here the dedifferentiated cells, preparatory to resorption, group themselves into rounded or spheroidal masses. (Fig. 1-*A*.) Nematocysts as well as numerous cell-fragments may be seen within these aggregates. Obviously there must be some change in the non-living mesoglea as well as the living endoderm before resorption of the modified ectoderm can proceed. Dedifferentiation, therefore, starts in the endoderm. These cells, apparently, break away from the walls of the tentacles and soon assume a globular form (Fig. 1, *B*).

They migrate down the lumen of the tentacle (Fig. 1, *B*). Now the mesoglea breaks or is resorbed (Fig. 1, *C*) and the endodermal elements apparently have little trouble in finding their way to the coelenteron. The cellular masses of ectoderm, spheroidal in shape and often with contained nematocysts, together with the above mentioned dedifferentiated endo-epithelial masses, may be found in the coelenteron as far down as the basal disc. Thus the surface of the tentacle is decreased. To use the language of Huxley and DeBeer ('23) in describing a similar phenomenon in *Obelia* and *Campanularia*, "The ectodermal cells may be compared with that of a rear guard, retreating yet always maintaining an unbroken front." These histological details serve as a final criterion for determining whether dedifferentiation and resorption have taken place. But with the aid of low magnification, one can see that, as resorption proceeds, the tips of the tentacles increase in diameter, and finally appear knobbed and the involved area becomes darker and darker, The endodermal cells lining the tentacles are normally highly vacuolated. These cells, however, appropriate relatively much food during the later stages of resorption.

It is certain that this dedifferentiated and resorbed tissue is used as food by the animal because nematocysts in various stages of digestion may be found in the epithelio-muscular cells in all parts of the endoderm. This confirms Kepner and Jester ('23) in their minor claim that the ingested parts were used as food; but Kepner and Jester were misled by the occasional biting off of the tentacles. Dedifferentiation and resorption are the usual reaction.

Since it was seen that both the cells of the ectoderm and the endoderm of the tentacles were almost exactly like those of the lateral walls of the basal one third of hydra, dedifferentiation and resorption was looked for in this basal region. It was found to occur in the case of the sectioned hydra recorded in culture number 5 (Fig. 2, *A*). No explanation is offered for dedifferentiation and resorption being found in the basal disc in this and no other case. It was noticed, however, that in this case resorption was not found in the tentacles. Resorption has not been reported before as occurring in the basal region prior to its inception in the tentacles and peristome. All other writers state that it starts at the tentacles and proceeds towards the base. The peristome is affected, according to them, after the tentacles have been removed. But this specimen showed dedifferentiation only in the basal region.

Green hydra reported in culture number 6 which was carried twenty-three days without food, showed histologically only slight resorption.

Rehm ('25) says that at the end of twenty one days the body of hydra subjected to inanition was reduced to a mere rounded form, which he calls, following Will and other investigators, "Reductionskörper" (§ 371). At other places he refers to these rounded hydras as presenting planula-like pictures ("planulaähnliches Gebilde, der Reductionskörper") (§ 382). We have carried brown hydra for twenty three days within the optimum hydrogen ion concentration. This polyp showed so little dedifferentiation and resorption that they could only be detected histologically. Under low magnification the living polyp, though reduced in size, appeared to be complete and have no broken surface. The brown hydra, as recorded in culture number 8, which was sectioned after sixteen days of inanition within the optimum hydrogen ion concentration, presented, while living, no evidence of dedifferentiation and resorption under low magnification. However, the histology of this animal shows frequent nematocysts in the coelenteron hence slight dedifferentiation and resorption must have taken place during the seventeen days of inanition. Examination on this day under the dissecting microscope disclosed no difference in appearance between the remaining hydras and the one sectioned. On the twenty third day another hydra from this culture was sectioned. From the histology of this polyp, it is seen that dedifferentiation and resorption which were shown in the histological examination of the hydra sectioned on the 17th day not only has ceased but the resorbed tissue has been digested by the polyp sectioned after twenty three days of inanition within the optimum range of hydrogen ion concentration. Similar phenomena have been observed for green hydras. For a green hydra, which had suffered 14 days of inanition at optimum hydrogen ion concentration showed slight dedifferentiation and resorption; while a second green polyp, from the same culture sectioned after twenty three days of inanition at optimum hydrogen ion concentration, showed no evidence of dedifferentiation and resorption.

Thus it appears that during inanition at optimum hydrogen ion concentration a crisis is reached after about two weeks. During this crisis slight dedifferentiation and resorption make their appearance. The resorbed material may supply sufficient nourishment to tide the polyp, now reduced in size, through a long period before a second crisis develops and compells the dedifferentiation and resorption of more tissue.

SUMMARY.

1. The optimum range of hydrogen ion concentration for both *Hydra viridissima* and *Pelmatohydra oligactis* lies within the range pH 7.8 and 8.0.

2. Polyps allowed to develop pronounced dedifferentiation and resorption in a high hydrogen ion concentration (low pH) were induced to completely restore their lost parts when the medium was altered to be within the optimum range of pH.

3. Hydras carried within the optimum range of pH were subjected to periods of inanition as great as twenty five days without showing any external evidence of dedifferentiation and resorption at the end of this period.

4. Histological preparation of polyps, kept for long periods without food at the optimum hydrogen ion concentration, show slight evidence histologically of dedifferentiation and resorption at a critical period. This critical period appears somewhere between ten and seventeen days after inanition within the optimum range of pH. Such microscopic dedifferentiation and resorption are not progressive; for after this critical period has passed no further histological evidence of dedifferentiation and resorption has been observed.

(*b*) This microscopic dedifferentiation and resorption usually appear at the tips of the tentacles; but in one case we have seen it involve the basal third of the polyp and not the tentacles.

5. Hydras subjected to long periods of inanition within the optimum range of pH accept food readily. There is, therefore, no evidence of depression given by these polyps.

6. Dedifferentiation and resorption are induced rather by unfavorable hydrogen ion concentration than by inanition.

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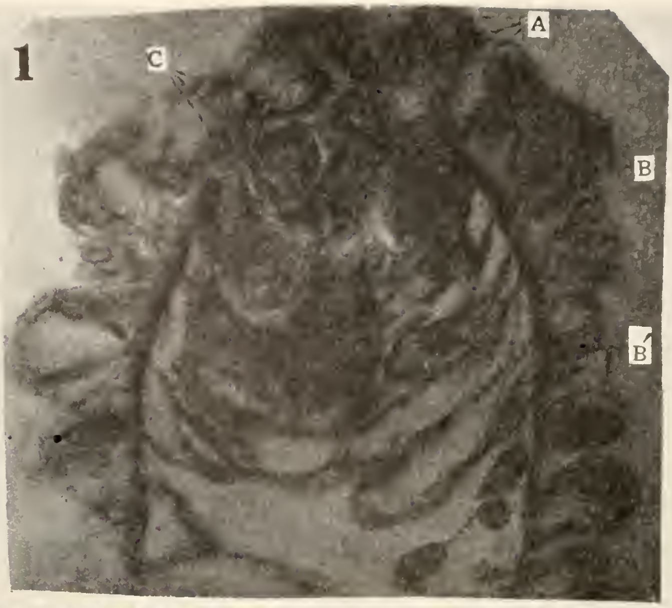
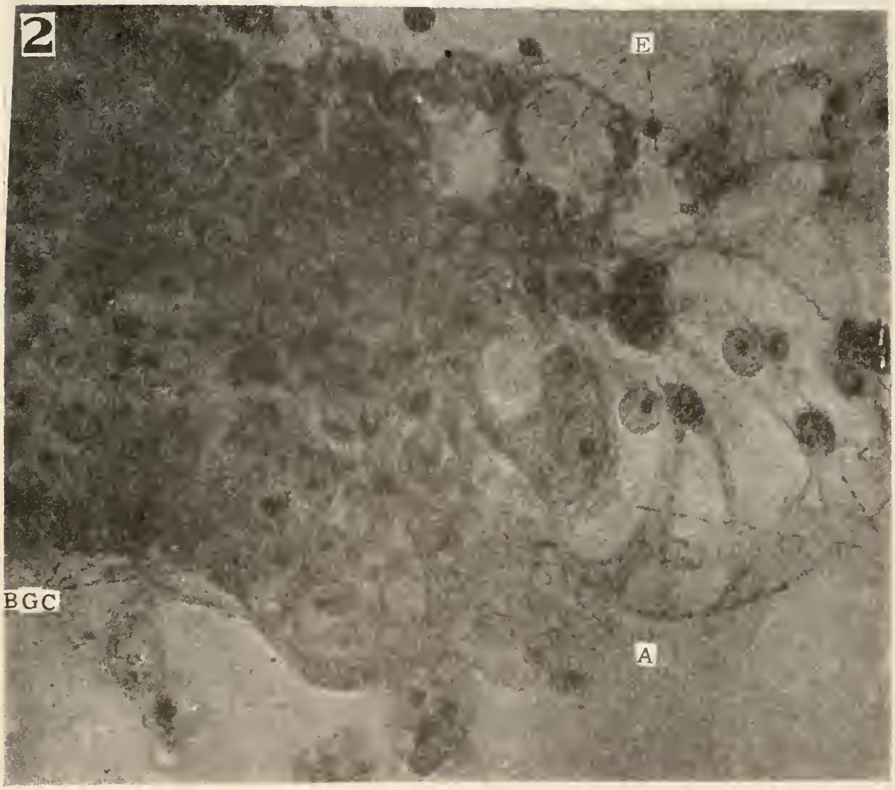
PLATE I.

Explanation of Figures.

FIG. 1. Longitudinal section of the free end of a tentacle of *Pelmatohydra oligactis* which had been starved twenty-four hours in spring water at pH 6.8. This shows the inception of dedifferentiation and resorption. The mesoglea has broken down at end of tentacle. Rounded masses of coalesced ectodermal cells are forming (*A*). Similar rounded masses of coalesced endodermal cells are forming (*B*); at *B′* we see a mass of coalesced endodermal cells having migrated towards the lumen of the tentacle; at *C* a mass of coalesced ectodermal cells is passing through the region of the broken down mesoglea. ×700.

FIG. 2. A longitudinal section involving a part of the basal disc of *Pelmatohydra oligactis*. (Culture number 5.) This specimen had been starved nine days within optimum hydrogen ion concentration. The inception of dedifferentiation and resorption is shown at *A*; *BGC*, basal disc glands cells; *E*, endodermal cells; *L*, lateral ectodermal cells. ×700.





W. L. THRELKELD AND S. R. HALL.

THE OCCURRENCE OF NUCLEAR VARIATIONS IN *PLEUROTRICHA LANCEOLATA* (STEIN).

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The occurrence of variations from the accepted type among the protozoa has received much attention in recent years, and a number of such cases have been reported, both of the artificially induced and spontaneously appearing sort. Most of the former have been of the "enduring modification" type, that is they persist throughout a longer or shorter period of vegetative division, but are eventually lost when conjugation or endomixis takes place. The latter may be divided into two classes. The first group would include the true mutations, of which the tetraploid *Chilodon* described by MacDougall (1925) is probably one of the best authenticated examples. In this case the mutation, which consisted in the possession of twice the usual number of chromosomes, combined with unusual size and certain other minor characteristics, persisted through both conjugation and division. To the second group would belong all other departures from normal, such as the production of monsters, the amicronucleate condition in infusoria, and various other unusual physiological and morphological characters which persist through division but tend to revert to normality eventually. Examples of this kind of variation are quite numerous. Among them may be mentioned the amicronucleate *Oxytricha* studied by Dawson (1919), the race of *Paramecium* which possessed extra contractile vacuoles (Hance, 1917), the rapidly-dividing race of *Didinium* reported by Mast (1917), and the sudden appearance of an *Arcella* having double characteristics described by Reynolds (1923). Since the latter investigator found that these abnormal characteristics could be diminished until a completely normal condition was reëstablished, or increased by selection of suitable individuals this last variation evidently belongs with those found by Jennings (1920) and Root (1918) to exist in *Difflugia* and *Centropyxis*, with this difference, however—the former occurred suddenly, while the latter were of lesser degree and appeared more gradually. More recently Dawson (1924) has reported the occurrence of a peculiar form of *Paramecium aurelia* which has been carried in culture for several years since. The abnormal character in this case consists of a "notched" condition which is definitely heritable, at least in ordinary asexual division.

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The present paper deals with variations in the number of both micro- and macronuclei in *Pleurotricha lanceolata*. Pedigreed cultures of this ciliate, which is a hypotrich belonging to the family Oxytrichidæ, were maintained for 18 months and studied mainly from the standpoint of the cytological changes occurring during conjugation and division, as described in a previous paper (Manwell, 1928).

The normal animal is shown in Fig. 1. It will be noted that it possesses two nuclei of each sort, and according to Stein (1858) who first described both the species and genus, the presence of two macro- and two micronuclei is a generic character. About two months before the culture was discontinued however, and while to all appearances it was in a very vigorous condition with division taking place very actively, individuals possessing only one macronucleus were noticed in some of the stained preparations. The micronuclear condition varied; in some cases there was only one and in others there were two as in normal individuals. Animals possessing the normal macronuclear complex but with three micronuclei have also been observed, and such changes are indeed not very uncommon, not only in *Pleurotricha* but in *Oxytricha* and other ciliates containing more than one micronucleus. But no individuals have been observed with only one macronucleus and more than two micronuclei. Fig. 2 shows an individual possessing but one nucleus of each sort in division, and in Fig. 3 a similar individual, differing only in having two micronuclei, may also be seen dividing. The next two figures show later stages in the division of such individuals, and in Fig. 6 a unimacro- and micronucleate animal is shown just after division.

From these figures it can be seen that division takes place in exactly the same way as it does in individuals having the normal nuclear complex, and that the variations are heritable, at least in ordinary vegetative fission. To settle this point still more definitely several lines were started from individuals possessing but one nucleus of each sort and followed for 10 days. At the end of that time these subcultures were lost by accident and other circumstances made it necessary to conclude the experiment, but stained preparations made from each generation showed clearly that the reduced number of nuclei was being passed from one generation to the next.

A careful examination of stained preparations has been made in an effort to discover whether the abnormal nuclear complex was accompanied by any other morphological changes, but apparently there were none. During the early stages of division however (about the stage shown in Fig. 2) it was frequently possible to distinguish animals possessing but one macronucleus from normal individuals in the same culture in a similar stage, for the bodies of the former were definitely broader about 1/3 of the way back from the anterior end and then tended to become narrower, while in the normal animals the entire middle third of the body was of a fairly uniform width. If there were any differences in size they were in favor of those individuals possessing but one nucleus of each sort.

No evidences of conjugation among these abnormal individuals was ever observed, but since as previously reported, conjugation occurred but rarely in all the cultures from start to finish of the experiment, not much stress can be laid on this point. Encystment was also not observed. Consequently it cannot be said whether such a variation as this would survive endomixis and conjugation, although it seems probable that in some cases at least, unimicro- and macronucleate conjugants might produce similar individuals.

In view of the work of Baitsell (1914), and the fact that conjugation in this species has been shown to result, at least when it occurs under cultural conditions favorable to vegetative division, in almost 100 per cent. mortality (Manwell, 1928) the question of the occurrence of such morphological variationsas herein described becomes of some practical importance. For obviously, if under favorable conditions multiplication by fission can continue indefinitely, then such changes might be perpetuated for a very long time in nature, as well as in artificial cultures. And if this is so account should be taken of the fact in the description of genus and species, since the number of nuclei, especially of the macronuclei, is a conspicuous character. If asexual reproduction can continue indefinitely then the sudden appearance of changes of the kind described would, for practical purposes, have the value of a mutation.

The occurrence of abnormal micronuclear conditions has been reported a number of times before, particularly with respect to the total absence of a micronucleus, and the presence of one or two supernumerary micronuclei is not very uncommon in species ordinarily possessing two or more, as already noted, but apparently the number of macronuclei is a much more constant character. The only instance in which a variation in the latter has been reported, to the author's knowledge, at least, is that given by Calkins (1926). Here he states (p. 579) that in early cultures of *Uroleptus mobilis* the number of macronuclei was almost uniformly 8, but as the age of the cultures increased individuals with a greater number of nuclei became common, until finally the number was nearly always 14 or 15.

SUMMARY AND CONCLUSIONS.

In a pedigreed culture of *Pleurotricha lanceolata*, a species o, hypotrich normally possessing two macro- and two micronuclei individuals with only one macronucleus and one or two micronuclei suddenly appeared, at a time when division was rapid and the culture apparently very vigorous.

That the difference in nuclear number was heritable, at least in asexual multiplication, was shown from stained preparations and pedigreed lines, and the fact that it has been shown that this species will live and divide normally apparently indefinitely under favorable conditions, without conjugation, makes it probable that such variations as have been described would continue for a very long time, and that animals with such peculiarities may be common in nature as distinct varieties.

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EXPLANATION OF THE FIGURES.

Magnification × 550; all drawings made with camera lucida

PLATE I.

FIG. 1. A typical vegetative individual.

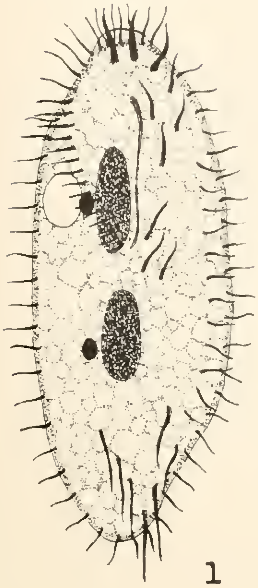
FIG. 2. An individual with one macronucleus and one micronucleus in a moderately early stage of division.

FIG. 3. A division stage similar to the above in an animal having two micronuclei, but only one macronucleus.

FIG. 4. A more advanced stage in an individual similar to the above.

FIG. 5. The final stage of division in a unimicro- and macro-nucleate individual.

FIG. 6. A daughter individual just after fission.



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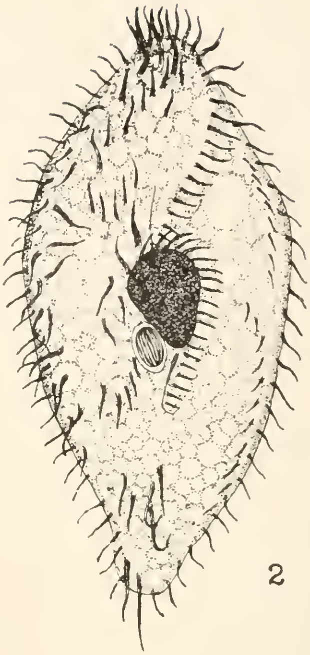

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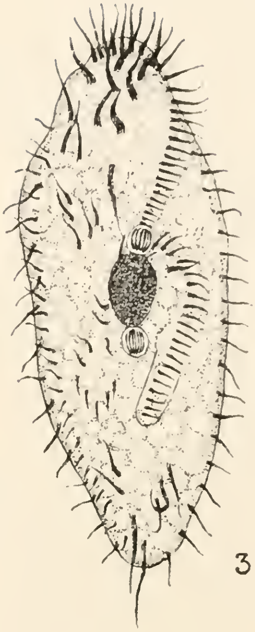

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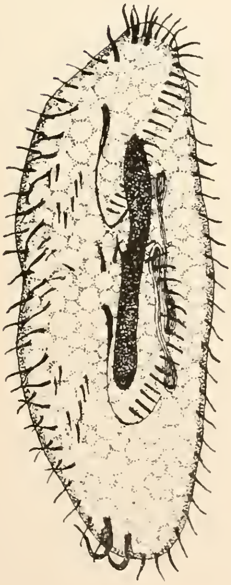

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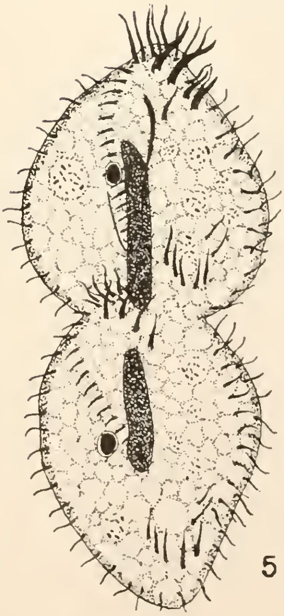

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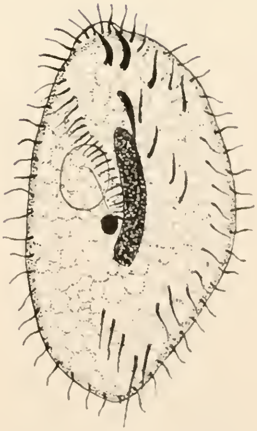

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REGINALD D. MANWELL

OBSERVATIONS ON THE LIFE HISTORY AND PHYSIOLOGICAL CONDITION OF THE PACIFIC DOG FISH (*SQUALUS SUCKLII*).

J. P. QUIGLEY.[1]

Incidental to an investigation of the reactions of *Squalus sucklii* to variations in the salinity of the surrounding medium (1) observations were made regarding the life history and physiological condition of this fish.

The fish were captured during the months of June, July and August of 1926 from the Straits of Georgia in the vicinity of Departure Bay, Vancouver Island, B. C. They were taken on a set line, the hooks of which were baited with pieces of salted herring. Most of the fish were obtained at a depth of about 30 meters, and they were generally caught near kelp beds. A sample of water taken at a depth of 30 meters in the region where many of the fish were taken was found by Lucas (2) to have the following characteristics; pH 8.4, temperature 10.3° C., density 1.0218, oxygen content 4.41 cc. per liter, sodium chloride content 27.37 gm. per liter.

Weight of Fish.—It was found that many of the factors associated with the weight of the fish could be emphasized by grouping the fish according to weight as has been done in Table I. Examination of this table shows that with the fish of lighter weight the two sexes are nearly equally represented, the number of males being slightly greater. As heavier fish are considered, the relative number of males shows a marked increase, then a sudden decrease so that in the weight divisions above 4,000 grams the males are entirely absent.

These results probably indicate that male fish with body weight over 4,000 grams do not exist in this locality during the summer. It cannot be definitely stated that the figures obtained with fish of lighter weight indicate the relative proportion in which the

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[1] From The Pacific Biological Station, Nanaimo, B. C., and The Department of Physiology and Pharmacology, University of Alberta, Edmonton, Alberta.

Table I.

Weight Limits (Grams).	Number of Fish Obtained.	Number of		Percentage.		Average Length (Cm.).	Average Increase in Length.
		Males.	Females.	Males.	Females.		
300–399 . . .	12	7	5	58	42	39.9	
400–499 . . .	15	9	6	60	40	43.6	3.8
500–599 . . .	16	11	5	69	31	45.7	2.1
600–699 . . .	13	8	5	62	38	48.4	2.7
700–799 . . .	5	5	0	100	0	52.5	4.1
800–899 . . .	7	4	3	57	43	53.8	1.3
900–999 . . .	5	5	0	100	0	54.7	0.9
1,000–1,499 .	22	20	2	91	9	60.3	5.6
1,500–1,999 .	11	9	2	82	18	69.2	8.9
2,000–2,999 .	30	26	4	87	13	74.9	5.7
3,000–3,999 .	13	5	8	38	62	83.3	8.4
4,000–4,999 .	16	0	16	0	100	90.5	6.2
5,000–5,999 .	16	0	16	0	100	91.6	1.1
6,000–6,999 .	4	0	4	0	100	95.5	3.9
7,000–7,999 .	1	0	1	0	100	99.0	3.5

two sexes occur, although such probably is the case. Since the fish were taken on a set line hunger or greed might conceivably be a factor in determining whether or not fish would take the bait. The stomach of fish captured usually contained much food, a fact which indicates that feeding for this fish is determined more by the availability of food than by hunger.

Out of 219 fish captured, 128 (58 per cent.) were males. Craigie (3) examined the fish obtained in the same region during July and August, 1925, and found that among 76 specimens 44 (60 per cent.) were males, while during December of 1925 by examining 117 specimens he found 47 (40 per cent.) males.

As was to have been expected, there is a comparatively definite relationship between weight and length of fish. The increase in length is rather steady though not entirely uniform as heavier fish are compared with those of lighter weight. It could not be shown that sex altered the relation of weight and length. There was a slight though inconstant indication that nonpregnant females were longer than pregnant females of the same weight. The longest fish captured measured 99 cm., the shortest 35.5. The heaviest fish weighed 7,550 grams and the lightest 300 grams. When increasing their weight 100 grams the smaller fish made an increase in length of approximately the same magnitude as did the larger fish when making a weight increase of 1,000 grams.

Pregnancy and Embryos.—Of the females captured, 43 per cent. carried embryos large enough to be readily noted in a cursory inspection. The lightest fish having embryos weighed 3,440 grams and was 85 cm. in length. These figures give an approximate minimum limit of the size of the mature female. Among the 50 females captured with a weight equal to or above 3,440 grams, 39 (78 per cent.) carried embryos.

Ford (4) quotes the conclusion of several investigators that *Squalus acanthias* breeds throughout the year and of other investigators that this species breeds only during certain periods. The results of his own investigations support the latter conclusion and tend to show that near Plymouth, England, specimens ready for birth would not be found earlier than the end of August. I found specimens of *Squalus sucklii* embryos at all times during the summer which ranged through all the sizes from the smallest to those with the umbilical scar healed completely and apparently ready for birth. This observation naturally suggests that in the vicinity of Nanaimo, *Squalus sucklii* breeds at all times of the year.

In any one parent, the embryos were of the same general size. A set of developing eggs was always found in females carrying embryos. The number of embryos obtained from 16 fish varied between 3 and 11 with an average number of 6.87. Although it could not be definitely stated that none of the embryos had been lost from the mother in the course of capture it is believed that this was a rare occurrence. No embryos were lost after the mother was taken from the set line and in most cases egg capsules still unruptured were obtained. In an examination of Squalus acanthias Ford (4) found that females of this species could carry as many as 11 embryos but the greatest number of pregnant fish carried only 3. In *Squalus sucklii* I found that embryos of both sexes usually occurred in the same uterus but there was no relation between the number of either sex, *e.g.* in one fish I found 6 females and 1 male, in another 3 males and no females. Of the embryos obtained 50 per cent. were males. This figure is to be contrasted with that previously noted for the fish of small size taken on the set line where a preponderence of males existed. A blue shark, *Prionace glance,* (identified by Professor J. R. Dymond) received at the Pacific Biological Station, August 19, 1926, was found to have 11 females and 8 male embryos all the same size nearly ready for birth.

Constitution of Shoals.—Throughout the period fish were being taken, the specimens obtained on any set line usually consisted of both sexes in approximately equal numbers and of all sizes. The conclusion was reached that the shoals consisted of both sexes and all sizes of fish or else the line had been visited within a few hours by several different shoals. It was also noted that the largest fish were usually taken at a greater depth (very near or actually on the sea bottom) than the smallest and it may be that the composition of shoals is in part determined by size. From his study of *Squalus acanthias*, Ford (4) concluded that for this species the mature males and females each form separate shoals while these shoals in turn are distinct from those composed of immature males and females together. I obtained fish in the same region throughout the summer. It is therefore likely that certain shoals inhabit this region during the entire season.

Summary.

1. Among the smaller fish males were slightly more prevalent than females. Males weighing more than 4,000 grams were not obtained. Females attain a much greater length and weight than males. The greater weight of the females was not always due to the presence of eggs or embryos.

2. A comparatively definite relationship exists between weight and length of fish. The relationship of length increase to weight increase for small fish is approximately ten times as great as for large specimens.

3. Of the mature females captured 78 per cent. carried embryos. This species apparently breeds throughout the year. The average number of embryos carried by the females is greater than six.

4. The shoals apparently consist of fish of all sizes and of both sexes. The shoals probably remain in the same region throughout the summer.

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ALGÆ OF PONDS AS DETERMINED BY AN EXAMINATION OF THE INTESTINAL CONTENTS OF TADPOLES.

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INTRODUCTION.

During the last few years a considerable amount of research has centered around the food taking of small fresh-water fish. This work has emphasized the dependence of small fish on algæ and in turn these fish as a source of food for the game fish. In reviewing literature the writer has found comparatively little scientific work on the feeding habits of the tadpole and frog.

The tadpole as well as the small fish is an indirect source of food for the human race. Tiffany ('22) states: "For most of the young fishes examined the complete story reads: 'no phytoplankton, no gizzard shad.'" It may also be said, no algæ, no tadpole.

The writer wishes to express her gratitude to Dr. Bruce D. Reynolds, who suggested this problem and who has greatly assisted by his advice and criticism in the preparation of this paper; also to Professor I. F. Lewis and Dr. E. M. Betts for helpful criticisms.

METHODS.

During the summers of 1927 and 1928 one hundred tadpoles and one hundred pond collections were taken from five ponds on the campus of the University of Virginia and in the surrounding vicinity. Two of the ponds measured approximately 250 ft. x 100 ft., one 150 ft. x 50 ft., one 100 ft. x 30 ft., and one 50 ft. x 20 ft. The ponds which were studied did not have active outlets.

Two examinations of each of these ponds were made during the summer of 1927 from July 15 to August 28, and two were made during the summer of 1928 from June 20 to July 5. Each collection from a pond consisted of five tadpoles [1] which measured from one and three-fourths inches to five inches long and five collections of sediment taken from the edges of the ponds. The tadpoles and pond collections were put in separate containers. Immediately after returning to the laboratory the tadpoles were killed and the intestines removed. Three slides were made of material taken from each digestive tract, one from the anterior and one from the middle regions of the small intestine, the third from the anterior region of the large intestine. A study of each of the slides was made under the high power of the microscope. The algæ from each region were identified and recorded. The pond collections were studied in a similar way. Three slides were made from each of the pond collections. The algæ from each slide were identified and recorded.

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[1] Of the 100 tadpoles used in these experiments, 94 were *Rana clamitans* and 6 *R. catesbeiana*.

During the summer of '27 the tadpoles were collected from the pond, and then the pond collections were made without any effort to correlate the position of the tadpole and the pond collection, but in the collections made during the summer of '28 a tadpole was caught and from the same place a pond collection was made.

The Problem.

The experiments presented in this paper were not undertaken primarily for the purpose of studying the food of tadpoles, but rather in order to ascertain if the algæ found in the alimentary tract of tadpoles can be relied upon as an index to the microscopic flora of the ponds in which the tadpoles are living. In other words, does the tadpole feed on different kinds of algæ or is it selective in its feeding habits? If not selective, is it as good a collector of algæ as the investigator interested in studying them?

Experimental.

In following up this problem observations were made on four collections, made at different times, from each of five ponds. The results obtained are shown in tabular form.

By referring to Table I. it will be seen that the number of species of algæ obtained from the intestine of the tadpoles exceeded the number obtained from the pond collections in every case except two, and in these instances they were the same—the pond collections being made where the tadpoles were caught.

Attention is also called to the relative number of algæ found in the intestines of tadpoles and the ponds from which they were taken, in large and small ponds (Table I.). It is evident that, when making collections from small ponds, the investigator is able to find most of the algæ present; whereas if the pond is a large one there is an appreciable difference between the number of species of algæ obtained by the two methods—the ratio being approximately 4: 3 in favor of the tadpole.

TABLE I.

SHOWING THE TOTAL NUMBER OF SPECIES OF ALGAE TAKEN FROM THE INTESTINAL TRACT OF FIVE TADPOLES AS COMPARED WITH THE TOTAL NUMBER FOUND IN FIVE COLLECTIONS MADE FROM THE SAME PONDS.

Size of Pond.	Collections Made during Summer of 1927.				Collections Made during Summer of 1928.			
	Jun. 15–Aug. 11.		Aug. 11–Aug. 28.		Jun. 21–Jun. 27.		Jun. 27–July 5.	
	Tadpole.	Pond.	Tadpole.	Pond.	Tadpole.	Pond.	Tadpole.	Pond.
250 x 100 ft......	50	32	59	39	63	49	58	48
250 x 100 ft......	54	42	45	37	44	44	56	49
150 x 50 ft......	52	46	47	44	65	56	59	46
100 x 50 ft......	35	30	63	50	56	47	47	41
50 x 20 ft......	35	30	46	44	47	39	44	44

As stated in a paragraph under Methods, three examinations were made of each pond collection and of each tadpole—one from the anterior region of the small intestine, one from the middle region of the small intestine, and one from the large intestine. Table II. shows the distribution of the species in different regions of the intestinal tract as compared with the total number found in the tadpole and the total number found in the pond collections. Usually more species of algæ were found in the anterior end of the small intestine, but there is not a great variation in numbers in the three regions. Most of the algæ found in the large intestine show slight evidence of having been acted upon by the digestive juices.

Even though the species of algæ found in the tadpoles outnumbered those in the pond collections, algæ which did not occur in the tadpoles' intestines were found in collections made from the pond. There was one exception, and in this case the tadpole and pond collection were taken from the same place. In this entire work only five species of algæ were found in pond collections

TABLE II.

SHOWING THE TOTAL NUMBER OF SPECIES OF ALGÆ FOUND IN DIFFERENT PONDS, THE NUMBER FOUND IN TADPOLES AND THE NUMBER FOUND IN DIFFERENT REGIONS OF THE INTESTINE.

A. S. Int., anterior end of small intestine; M. S. Int., middle region of small intestine; A. L. Int., anterior end of large intestine.

Pond.	Tadpole.	A. S. Int.	M. S. Int.	A. L. Int.
29	36	23	22	20
30	34	22	16	19
35	44	23	24	19
33	33	24	19	19
30	45	21	24	31
28	33	28	26	21
31	40	26	20	24
32	31	19	14	14
27	50	26	21	36
16	34	18	13	16
25	35	29	12	16
18	35	26	14	22
35	34	21	26	16
24	36	21	21	27
32	38	21	16	21
27	36	20	22	22
32	34	31	15	18
25	38	22	21	25
24	32	18	18	19
36	49	32	26	30

TABLE III.

COLLECTIONS MADE DURING SUMMER OF 1927.

Total Number Species from Both Sources.	Percentage of Those Found in Tadpoles.	Percentage of Those Found in Pond.	Total Number Species from Both Sources.	Percentage of Those Found in Tadpoles.	Percentage of Those Found in Pond.
50	86.20	55.17	70	82.85	55.71
68	79.32	61.76	58	83.10	63.79
70	74.28	65.71	57	82.62	77.19
45	77.77	66.66	68	93.64	73.23
37	94.59	81.08	53	86.79	75.28

COLLECTIONS MADE DURING SUMMER OF 1928.

67	94.03	73.13	64	95.31	77.50
50	88.	88.	58	96.55	84.48
66	98.48	84.84	62	95.17	74.19
60	94.33	78.33	56	100.	83.91
48	97.91	81.25	47	93.61	93.61

Showing total number of species of algæ taken from each pond, including the percentage of those obtained from tadpoles and from pond collections.

which were not also observed in the tadpoles. Evidently these species were very rare, for only one was encountered the second time. The fact that these algæ were not found in the tadpoles does not indicate, therefore, that the tadpoles refuse to eat them.

The variation in percentage of algæ from the two sources is less when pond collections and tadpoles are taken from the same place. This may be seen by referring to Table III. The pond collections made during the summer of 1928 were taken from the immediate vicinity in which the tadpoles were caught, while those made during the summer of 1927 were taken without regard to this matter.

Summary.

It is a well known fact that tadpoles feed on microscopic plants. The importance of this animal as a collector of algæ is clearly demonstrated. In comparing the intestinal contents of one hundred tadpoles with pond collections made from the same ponds, the number of species of algæ obtained from the tadpoles exceeded the number obtained from the collections in every case except two; and in these instances, they were the same. It may be stated, therefore, that an examination of the intestinal contents of tadpoles affords one of the best and easiest methods of determining the species of algæ present in ponds. This is especially true in large ponds, and applies particularly to the phytoplankton.

In this examination one hundred and seventy species and varieties of phytoplankton were found. Of this number, one hundred and sixty-five were encountered in the intestines of tadpoles.

Conclusion.

1. The food of green-frog tadpoles consists chiefly of algæ.

2. The algæ from pond collections and from the intestinal contents of tadpoles taken from the same ponds do not differ as much in small ponds as they do in the larger ones.

3. The anterior region of the small intestine is considered to be the best region for making examinations for algæ.

4. The species of algæ taken from the intestines of tadpoles constituted, on the average, 89.73 + per cent. of the total found.

5. An examination of the intestinal contents of tadpoles affords one of the best and easiest methods of obtaining a collection of algæ from ponds.

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FURTHER OBSERVATIONS ON THE CHEMICAL COMPOSITION OF WOODS HOLE SEA WATER—THE CHLORINE CONTENT AND SALT ANALYSIS.

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From time to time we have had occasion to make further observations on the sea water at Woods Hole since the publication of the original analysis (1). Though not in any sense complete it is believed that the following data may prove useful and therefore they are presented.

It should be pointed out that our aim has been always to select methods of analysis which would adapt themselves to the use of relatively small fluid volumes, as only in this way can they become applicable to the investigation of physiological and biological problems. From the large number of analyses of sea water tabulated by the Hydrographic Laboratory of Copenhagen, Knudsen, Dittmar (2) etc., further data of this kind have oceanographic interest but little more. There has, therefore, been made a conscious attempt to utilize more sensitive methods which require small samples for analysis, albeit the absolute values *may* not be quite as accurate.

DETERMINATION OF CHLORINE.

Since many physiological activities are sensitive to slight changes in the tonicity of the surrounding medium it seemed of interest to determine whether the chlorine content of the Woods Hole sea water varied to a significant degree from day to day. The method employed was as follows: Standard $AgNO_3$ was made such that 1 cc. was equivalent to 10 mg. chlorine. This was standardized against pure NaCl since it has been shown by Thompson (3) that this salt may be substituted for standard water from the Hydrographic Laboratory. The $AgNO_3$ was kept in the dark in a glass stoppered brown bottle and the standardization repeated at the end of the series of determinations. The method, thereafter, followed in detail that presented by the Association of Official Agricultural Chemists (4). The burette used was of 50 cc. capacity, standardized by the Bureau of Standards, Washington. 15 cc. samples of sea water were measured with a standardized pipette and diluted with distilled water to 35 cc. before titration.

Samples were taken from the laboratory tank. This tank is fed by water taken about 125 feet from shore. The other samples were taken from surface water as follows: (1) Buzzards Bay one half mile North of Robinson's Hole. (2) Cuttyhunk 300 feet from shore on the "Sound" side. (3) Tarpaulin cove one half mile out in the Sound; water 80 feet deep. (4) East of Nobska; water 28 feet deep.

Duplicate titrations were made and it may be said that these determinations but rarely disagreed.

The temperature was taken with not great accuracy, employing a standard 50 degree laboratory thermometer. Such slight changes as observed during these observations were not considered significant.

Grams of chlorine per kilogram were calculated from Thompson's empirical formula—

$$Cl_w = 0.008 + 0.99980\ Cl_v - 0.001228\ Cl_v^2$$

where Cl_w = grams of Cl per kilogram and Cl_v = grams Cl per liter at 20° C. A graph prepared by using the more common range of Cl contents was found useful.

The salinity—defined as the weight in grams of all the salts dissolved in a kilogram of sea water, after the carbonates have been converted to oxides, the Br and I have been replaced by Cl and the organic matter has been completely oxidized—was calculated from the relation derived by Knudson—

$$S/oo = 0.030 + 1.8050\ Cl_w$$

Of course it must be recognized that this is only an approximation, as Giral (5) has emphasized.

During these observations it should be stated that the weather was in general extremely bad, rain alternating with fog for dis-

Table I.

Chlorine Content of Woods Hole Sea Water During the Summer of 1928.

Date.	Source.	Temperature.	Grams Cl per Liter.	Grams Cl per Kilogram.	So/oo.
July 16...	Laboratory tank	21 degrees	17.80	17.42	31.47
" 18...	" "	22 "	17.80	17.42	31.47
" 21...	" "	21 "	17.86	17.48	31.58
" 23...	" "	21 "	17.77	17.39	31.42
" 26...	" "	21.8 "	17.77	17.39	31.42
" 28...	" "	21 "	17.86	17.48	31.58
August 1 .	" "	20.5 "	17.80	17.42	31.47
July 17...	Buzzards Bay..	20 "	17.93	17.54	31.69
" 17...	Cuttyhunk	20 "	18.00	17.60	31.79
" 21...	Off Tarpaulin Cove	20 "	17.93	17.54	31.69
" 21...	East Nobska	20 "	17.70	17.32	31.27

agreeably long intervals. The results, do not show any very marked changes in the Cl content of the water but it is altogether possible that a dry summer may increase the Cl content. Samples taken from other points along the uneven coast of Woods Hole show more evident variations, as was to be expected.

Sea Salt Analysis.

Samples of the dried sea salt taken from the laboratory tank during the summer of 1926 have been analysed, employing the classical methods as given in the Bulletin of the Official Agricultural Chemists (4) and by Scott (6). Though not complete, these data are presented, as they may be found useful.

Sea Salt of Woods Hole.

	Percentage.	
	No. 1	No. 2.
Sodium	30.68	30.49
Magnesium	3.31	3.48
Calcium	1.27	1.12
Silica	0.014	0.018
Phosphate	Trace	Trace
Nitrate	Trace	Trace

The above analyses would tend to confirm the suggestion made in our former paper that the Kramer-Gittleman direct method for the determination of sodium, while very convenient for relative data, may give an absolute value which is low. One must remember, however, that using the Haywood and Smith Method

(7) or that of Dittmar the sodium determination comes out low, as has been the universal experience of analysts. The values are then corrected by employing Dittmar's method (2) of "total sulphates." The older methods for sodium determinations are so cumbersome (as reference to Dittmar's article will show) that there is still some doubt as to the accuracy of the results.

During the Summer of 1928 we have again confirmed Atkins' (8) and Harvey's (9) work on the nitrates and phosphates. Samples of the Woods Hole water showed only the smallest trace of NO_3 and PO_4 during July 1928, the time at which our analyses were made this year. This change is, as they have shown, due to seasonal variations in the plankton.

Summary.

1. The chlorine content of Woods Hole sea water has been examined over a three-week period and shown not to vary within any large range.

2. Analyses of the sea salt are presented.

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THE PRECIPITATION OF CALCIUM AND MAGNESIUM FROM SEA WATER BY SODIUM HYDROXIDE.

ELEANOR M. KAPP.[1]

In the course of an investigation into the modification of sea water for use as a perfusion medium (Kapp, '28), it became necessary to know something of the relative amounts of calcium and magnesium precipitated by sodium hydroxide. Haas ('16) suggested that the first flat portion of his titration curve for sea water was coincident with the precipitation of Mg as hydroxide, the second with that of Ca. That this was a reasonable assumption is further suggested by the solubility product constants for the hydroxides of Mg and Ca, which are 1.2×10^{-11} and 4.1×10^{-6}, respectively (Johnston, '15). To obtain more exact information concerning this behavior of Mg and Ca, the following experiments were run on sea water taken from the English Channel outside the Plymouth breakwater, and from Great Harbor, Woods Hole, Mass.

Graded amounts of 10 normal NaOH (practically carbonate-free [2]) were added to 100 c.c portions of sea water. The flasks were stoppered and the contents thoroughly mixed. The supernatant fluid was filtered off as soon as the precipitate had settled somewhat (within four hours in all cases), and Ca and Mg were determined in separate samples of the filtrate. Ca was precipitated as oxalate from 25 cc. samples according to McCrudden's ('09) method, and allowed to stand in the refrigerator for at least 18 hours. The oxalate, after washing, was determined with permanganate. The Mg determinations were carried out according to the method of Willstätter and Waldschmidt-Leitz ('23) on duplicate 5 cc. samples from each filtrate. Values for total Ca and Mg were obtained by the same techniques from samples of untreated sea water, and show good agreement with the figures compiled by Clarke ('24) for sea water from a wide range of sources.

[1] From the Laboratory of the Marine Biological Association, Plymouth.

[2] Made up from the filtrate of a 50 per cent. solution in which the carbonate had been allowed to settle.

The behavior of Mg and Ca was investigated by Irving[1] ('26), but major emphasis was placed by him on equilibria within the biological limits of alkalinity. An extension of these investigations and an explanation of certain discrepancies which were encountered follow.

The data for Mg for Plymouth sea water are given in Fig. 1, and roughly agree with my results obtained on Woods Hole sea water by a less reliable technique. The curve for the precipitation of Mg as drawn by Irving is inaccurate, as owing to the scarcity of his points he completely missed the plateau. Fig. 1, however, substantiates the points he did determine.

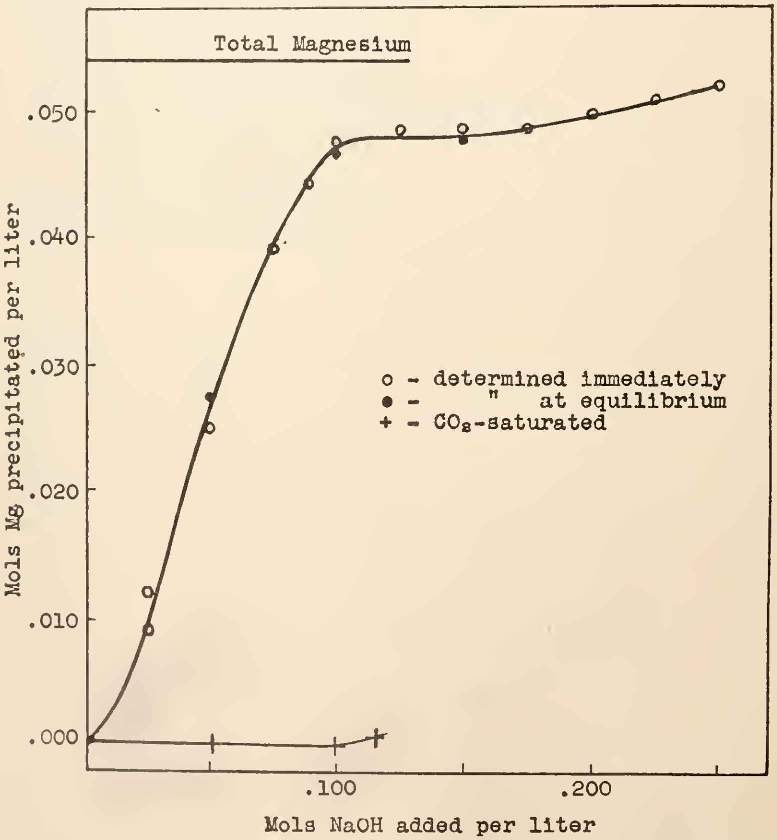

FIG. 1. The precipitation of Mg from Plymouth sea water in relation to the amount of NaOH added.

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[1] Unknown to me when this work was undertaken.

The data for Ca show that the results may be considerably modified by a slight variation in procedure. The Ca curves plotted as hollow circles in Figs. 2 and 3 both differ markedly from the one obtained by Irving. His technique was substantially the same as mine, with the exception that his original samples of sea water, after the NaOH had been added, were shaken for 24 hours instead of being filtered at once, so that equilibrium was insured. Since $CaCO_3$ tends to remain supersaturated, it was suspected of being the cause of the discrepancy. A control experiment was therefore set up, in which the NaOH was added very slowly as a normal (instead of 10 normal) solution, in order to avoid local high concentrations of hydroxide, and the stoppered mixtures were allowed to stand with occasional shaking for one week. At the end of this time they were filtered and analyzed.

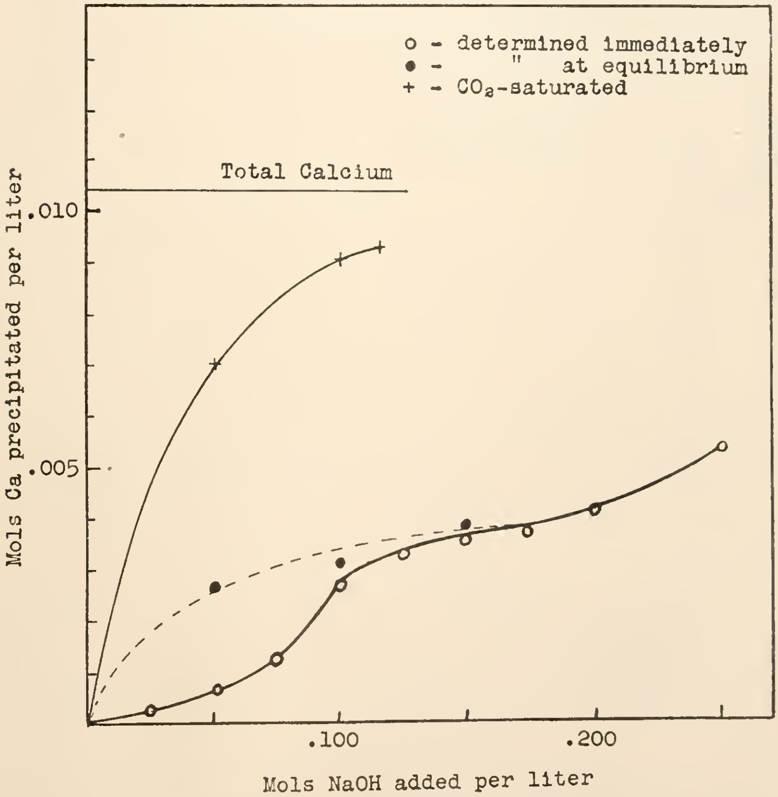

FIG. 2. The precipitation of Ca from Plymouth sea water in relation to the amount of NaOH added.

The Ca curve thus obtained differs from the first ones, this time confirming the results of Irving. Its points are shown in Fig. 2 as black circles. The difference between the two curves is therefore due only to the slowness with which $CaCO_3$ is precipitated, and can be controlled by taking the time factor into account. The same situation does not exist in the case of Mg, as can be seen from the black circles plotted in Fig. 1, which coincide with the original curve.

The effect of increasing the amount of carbonate was obtained by saturating several samples of sea water with CO_2 before the addition of the alkali. Increasing quantities of normal NaOH were then added very slowly, to allow the gelatinous precipitate which formed to redissolve, until the third sample, to which 11.5 cc. had been added, remained cloudy. The mixtures were aërated to drive off excess CO_2, and allowed to stand in contact with the atmosphere for one week. During this time a crystalline precipi-

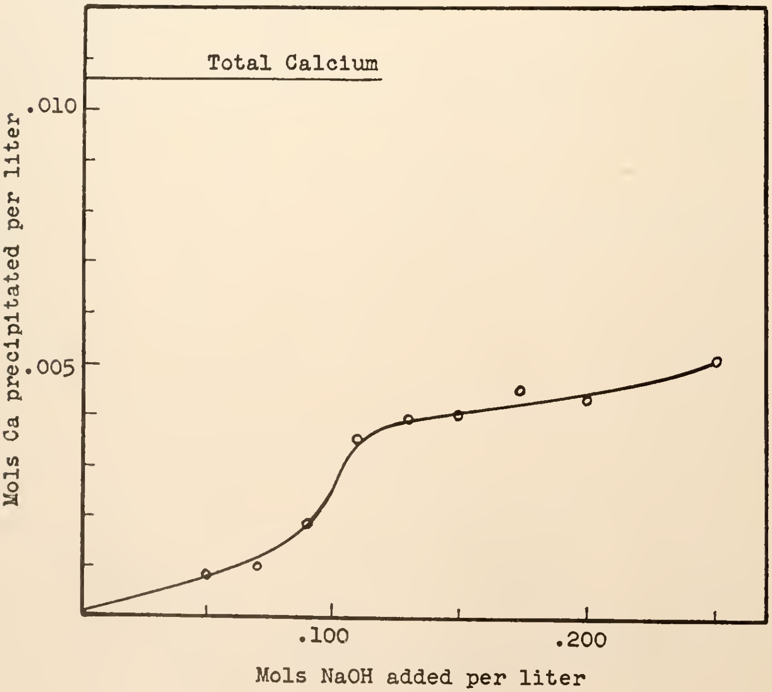

FIG. 3. The precipitation of Ca from Woods Hole sea water in relation to the amount of NaOH added.

tate had formed, and the solutions were filtered and analyzed as before. The results are shown by the crosses in Figs. 1 and 2, and are strikingly different from the other precipitations. In this case the addition of a small amount of alkali precipitates only the Ca, while the Mg is affected by larger amounts.

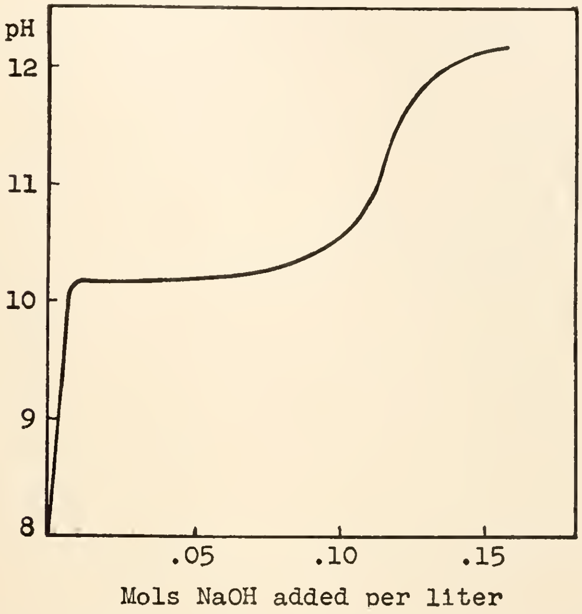

FIG. 4. The effect of NaOH on the pH of sea water (after Haas).

With reference to the reason for the shape of the Haas titration curve (Fig. 4), it is clear that Haas' own statement, mentioned previously, must be modified somewhat. As he suggested, Mg is precipitated rapidly by NaOH over the range where his titration curve shows a plateau. At a region corresponding to the addition of 0.1 mols of NaOH per liter of sea water, the titration curve begins its second rise, and the Mg curve flattens out. A small amount of Ca, however, is precipitated throughout, owing its first precipitation to the insolubility of the carbonate,[1] which is intermediate in this respect between Mg and Ca hydroxides.

I am deeply indebted to Dr. E. J. Allen, F. R. S., of the Marine Biological Association, Plymouth, for facilities extended to me during this investigation. I also wish to thank Prof. M. H. Jacobs and Mr. H. W. Harvey for their helpful interest.

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[1] $K_{S.P.} = .98 \times 10^{-8}$ (Johnston, '15).

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FURTHER OBSERVATIONS ON THE EFFECT OF HIGH FREQUENCY SOUND WAVES ON LIVING MATTER.

E. NEWTON HARVEY, ETHEL BROWNE HARVEY AND
ALFRED L. LOOMIS.[1]

Interest in the biological effects of very high frequency sound waves started with the investigations of Wood and Loomis (1) who devised methods for producing intense "supersonic" vibrations and described many of the phenomena connected with them. The reader is referred to this paper for a description of the two kilowatt generator and methods of working with the waves. The apparatus was of such high power and the sound waves of such great intensity as to produce considerable heating. It seemed highly desirable in working with cells to reduce the heating effects of the vibrations, and to observe the cell with the microscope while being radiated. After many attempts to use the high power oscillator as the source of the waves and to lead them to the material on the stage of a microscope along capillary rods and tubes, a low-powered apparatus was decided upon as the most convenient for the purpose. This has previously been described by Harvey and Loomis (2) together with some of the effects of these supersonic waves on living organisms, cells and tissues. The outfit consists of a 75 watt high frequency oscillator and a quartz crystal whose vibrations, produced in the electric field by reversal of the piezo-electric effect, travel through any medium in contact with the crystal. A frequency of 400,000 per second was used and the material mounted directly on the crystal which served as a microscopic slide. Schmitt, Olson and Johnson (3) have also described various biological effects using a 250 watt generator with crystal immersed in xylene. They lead the sound waves along a rod of small diameter ending in a microneedle, which could be inserted into the material to be studied.

Some additional effects have been recently observed with our

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[1] From the Marine Biological Laboratory, Woods Hole, the Physiological Laboratory, Princeton University, and the Loomis Laboratory, Tuxedo Park, N. Y.

75 watt outfit in its original form and also modified to use higher frequencies by changing the capacity, inductance and crystal. The new quartz crystal was a spectacle lens which happened to be cut in the proper direction, kindly loaned by Dr. Kenneth Cole. The natural frequency of this crystal was approximately one and one quarter million per second. Its thickness varied from 1 to 1.8 mm. and consequently the distance between the tin foil electrodes, was much less than in the original 7 mm. crystal, giving a far more intense electrical field and greater effects. A few experiments have been made with a 2.25 million crystal which vibrates well and gives the same effects with Elodea as the 1.25 million. A 6 million crystal, 0.45 mm. thick, does not vibrate strongly. We are at present engaged in increasing the frequency to the highest point possible to see how biological effects will vary with the frequency.

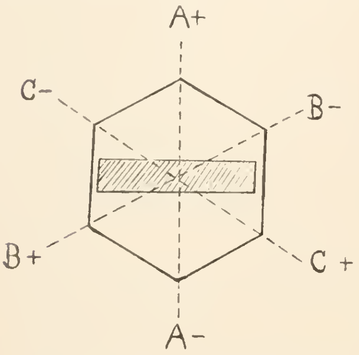
A convenient means of finding the resonant frequency of the crystal is to set it up between the two tin foil electrodes with holes in their centers (to allow light to pass for microscopic observation) and then place a drop of water on the crystal. At various settings of the condenser the water will be violently agitated and broken up into fine droplets like steam. Low melting point crystals placed in the water show that the temperature does not rise but that the "steam" is mechanically formed, as observed in various ways by Wood and Loomis (1), and not a condensation from vapor. The exact specifications for an oscillator giving various frequencies will be found as an appendix to this paper.

If an Elodea leaf covered with a cover slip is mounted on a crystal whose resonant frequency is 400 kilocycles, and relatively weak (by reducing filament current) sound waves sent through the leaf, it can be observed under the microscope that only certain areas in the leaf show the characteristic whirling of the chloroplasts described in our previous paper (2). The areas do not correspond to any position on the crystal but to some peculiarity in the leaf, as moving a leaf to a new position over the crystal does not necessarily change the areas of marked whirling. These areas of whirling are most marked where air bubbles, which vibrate strongly, are caught under the leaf and where the cells are several layers in thickness, near the midrib (which also contains air in intercellular spaces). Part at least of the condition for rapid whirling is the distance of the leaf from the crystal. By attaching the coverslip to a mechanical device for adjusting its distance from the crystal, the amount of water between coverslip and crystal can be varied and a slight change in this layer of water will cause whirling in a given area to start or to stop. These effects are no doubt due to interference of two sets of sound waves resulting in complicated interference patterns with nodes and internodes. Fine particles like red blood corpuscles suspended between crystal and coverslip can be observed to collect in nodes forming such a pattern. The chloroplasts in Elodea cells cannot do so since they are restricted in movement by the cell walls but in a region which happens to be an internode, they will undergo rapid whirling movements. The part played by an air bubble in causing rapid whirling is no doubt to offer a reflecting surface around which interference pattern and nodes appear. The whirling itself is probably due to the radiation pressure of the sound waves as they pass through the cells.

Another phenomenon regularly observed is a variation in the rate and character of the whirling as the variable condenser is changed to vary the frequency. For instance, over a range of 10 kilocycles, there appeared maximum whirling in a given area of the leaf at 407, 409, 410.4, 412.5, 415, and 417 kilocycles, *i.e.* a maximum approximately every 2 kilocycles, with no whirling or very slow whirling between.

In order to understand the changes in whirling motion imparted to the biological material placed upon the quartz as the frequency is varied, it is necessary to digress a moment and consider the forces acting upon an oscillating quartz disk. As is well known, a natural quartz crystal has three electric axes perpendicular to the optic axis. (See Fig. 1.)

The disk is cut as indicated by the shaded portion, *i.e.* so that one of the electric axes shall be perpendicular to the plane of the disk. If pressure is applied to the side of the disk corresponding to $A-$ a negative charge will accumulate there, while correspondingly if a negative charge is applied there without pressure the disk will contract as if the equivalent pressure had been applied. The same holds true with positive charges on the $A+$ side. On the other hand, when a positive charge is placed on the negative side and a negative charge on the positive side, the crystal will expand. A rapid alternation of charges causes the crystal to oscillate and as a first approximation the crystal can be considered to be an oscillating rigid piston. This would be rigorously correct if the disk were perfect and infinitely large but with a finite disk the forces are not symmetrical near the edges and a complex wave pattern is formed in the crystal. This can easily be seen by first considering a point *O* on the surface of the crystal near the center (Fig. 2). If a unit negative charge is placed on the under surface with the corresponding positive charge on the



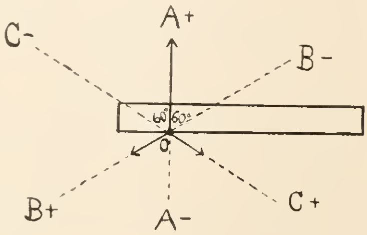


FIG. 1. Quartz crystal (shaded) cut perpendicular to optic axis. Electric axes indicated by *AA*, *BB*, *CC*.

FIG. 2. Vectors showing forces in point *O* in crystal.

upper, the crystal at "*O*" will tend to contract along the axis *OA* + and expand along the axes *OB* — and *OC* —. The intensities of these forces are directly proportional to the potential gradiants along the respective axis. The forces along *OB* — and *OC* — are therefore only half as great as along *OA* — since the distances through the crystal along these axis are twice as great as along the axis *OA* — (the angles between the axis being 60°). The vector resolution of the forces *OC* — and *OB* — along *OA* — shows that they are equivalent to a force opposed to the force along *OA* — and of magnitude equal to one half that of *OA* —. The vector equivalent of all three forces is therefore a single force along *OA* — equal to half of what that force would be if the forces along the axes *OC* — and *OB* — were not present.

This symmetry does not maintain however near the edges of the disk. Consider the point *Q*, Fig. 3. The axis *QB* is not in the crystal at all. The resolution of the forces along $QA-$ and $QC-$ gives a force along QX equal to the force along $QA+$ multiplied by $\frac{1}{2}\sqrt{3}$. It is clear, therefore, that the forces near the edges are not symmetrical and tend to produce distortions which travel in waves across the disk.

A second system of forces are also acting on the disk. As the quartz contracts normally to the surface it expands parallel to the surface (this effect is best seen in a rectangular plate). Thus the series of longitudinal waves create interference patterns with the traverse waves.

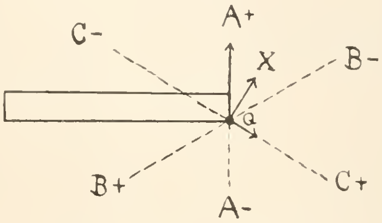


FIG. 3. Vectors showing forces in point Q in crystal.

Thirdly, it has been shown that even with a perfect quartz crystal the intensity of the piezo electric effect varies in different parts of the crystal. Dye has photographed the distortions produced in the interference fringes of an interferometer when one of the plates is an oscillating quartz disk. These photographs show most beautiful and complex patterns which showly drift across the plate when the frequency is slowly changed.

Fortunately in biological investigations under the microscope good use can be made of these complex patterns. Thus, without changing the position of the specimen on the crystal, one can by merely changing slightly the frequency, cause these patterns to shift so that any particular part of the specimen can be made to experience forces of varying magnitude and direction. Thus in a particular cell of *Elodea* the chloroplasts can, at will, be made to rotate slowly or rapidly, clockwise, or counter clockwise, in one vortex or in a series of vortices, while merely watching the specimens under the microscope and observing the effects produced as the frequency is slowly varied.

By increasing the intensity, the leaf of *Elodea* can be agitated so violently that the chloroplasts themselves are broken up into a fine green emulsion which completely fills the cell. This effect is not due to heating, since crystals of ethyl stearate, melting at 30–31° C. and placed on the *Elodea* leaf, are not melted even after 15 minutes, nor does slowly heating *Elodea* leaves bring about this effect. Neither is it due to possible mechanical rupture of the cellulose wall or mixing of the vacular sap with the chloroplasts, since unrayed cells can be crushed with a needle and their chloroplasts do not break up in this characteristic manner. The emulsification is caused by the tearing action of the sound waves.

Perhaps it should be emphasized at this point that these effects are all due to high frequency sound waves and not to any influence of the oscillating electrical field, as control experiments using glass plates of a size similar to and replacing that of the quartz crystal have shown.

Some of the more interesting of the effects observed with the spectacle lens crystal and frequencies of 1,250 kilocycles are as follows:

Amœbæ proteus or *dubia*,[1] moving along the surface of the crystal are not particularly affected by an intensity that causes the inclusions in small vacuoles of the *Amœba* to rotate on their axes. Higher intensities cause a mild whirling of the more liquid regions of the *Amœba* followed by rupture of the pellicle on one side and extrusion of the contents which join the general whirl of fluid in the medium. There is a tendency for the *Amœba* to move more rapidly during the raying as if the endoplasm became more liquid. After this there is a sudden change in direction of movement.

Both unfertilized and fertilized sea urchin (*Arbacia*) and starfish egg are violently agitated and may spin around. The jelly is torn off and the fertilization membrane may be broken. The eggs are thrown into rows or clumps and eventually cytolyze either partially or completely, the cytolysis taking place on one side and sometimes within the fertilization membrane. There is no movement of materials inside the egg caused by raying, as can be determined with certainty by using centrifuged eggs, the stratified layers remaining intact until cytolysis takes place. Cytolysis may take place from any of the stratified layers. However, if the unfertilized centrigufed eggs are placed in diluted sea water (40 distilled water to 60 sea water) and thus made less viscous, the inside may be made to whirl. The whirling takes place in the lighter layers, including the oil, clear and granular layers, but only along the edge of the pigment layer, most of which remains intact. The oil drops tend to remain together, but the clear and granular layers become mixed and after ten or fifteen minutes of whirling a clear zone can not be distinguished. The direction of rotation may be reversed instantly by a slight change in frequency. Sometimes instead of a whirling of the protoplasm, there is a streaming of granules similar to that of an *Amœba*. No whirling of protoplasm nor movement of granules has been observed in eggs put in dilute sea water and not centrifuged. This may be due to the fact that the less dense material, where the whirling takes place is not separated out from the heavier pigment granules.

The asters are quite unaffected by raying. Cleavage furrows will come in normally during raying, even when the egg is violently agitated. When an egg has been slightly cytolyzed by raying we have observed that the furrow may come in at the proper place. Eggs in the two or four cell stage may have one or two blastomeres cytolyzed and the others unaffected.

Arbacia plutei swimming slowly are paralyzed by a momentary raying, presumably because the cilia are torn off. Otherwise they look uninjured but more prolonged treatment or greater intensity will tear them to pieces, leaving only fragments of the skeleton behind.

The gill cilia of *Mytilius* do not seem to be affected by violent agitation of the sea water about them, until the cilia and gill filaments are actually torn to pieces.

Pigment cells well-expanded in the scales of *Fundulus* are not affected, although the scales are rapidly agitated as the waves impinge upon them.

Frog abdominal muscles mounted on the crystal show no contraction or movement although air bubbles and blood corpuscles on top of the muscle tissue whirl rapidly. The waves must have passed through the muscle tissue to reach the air bubbles and corpuscles.

Fragments of the rays of the ctenophore, *Mnemiopsis*, containing luminous material, mounted on the crystal in the dark and waves passed through, are agitated and occasionally luminesce. There is no continual luminescence which can be attributed to the waves but only the sporadic luminescence connected with sudden movement of the fragment such as can be obtained on jarring the table containing fragments of *Mnemiopsis*, even when not exposed to high frequency sound waves.

Fundulus embryos within the egg, with beating hearts, subjected to waves of an intensity to agitate the eggs but not so great an intensity as to interfere with observation of the heart beat show no marked effect upon the character of the beat or circulation. In fact only the effect observed was a slight increase in rate during raying which can be accounted for by a slight increase in temperature, that undoubtedly occurs when these high frequency waves carrying considerable energy, are absorbed by the medium. The embryos were rayed 1 minute and then not rayed for one minute while the heart beats were counted. In four experiments the rates were: Rayed—148, 157, 140, 132; unrayed—140, 148, 122, 122, respectively. The average increase in rate was about 8 per cent., which can be accounted for from the known effect of temperature on the heart beat of *Fundulus heteroclitus*,[2] by a rise of temperature from 22° C. to about 23° C.

Perhaps it should be emphasized again from the experiments on muscle, heart, luminous cells and chromatophores that there is no stimulating effect of these waves similar to the stimulation by electrical or sudden mechanical disturbance.

Fertilized *Fundulus* eggs mounted on the crystal can be very violently agitated and the oil drops and granules within made to dance. The yolk can be thoroughly stirred and the surface of the protoplasm can be observed to move and bend. Dr. Elmer Butler has carried these eggs to the point of hatching and finds the development and the embryos normal. If the agitation has continued so long as to burst the protoplasmic surface development does not proceed. An intensity of raying which does not destroy the surface has no effect on development while a slightly greater intensity results in dissolution and cytolysis.

Study of a large number of cells and tissues, some of which are recorded above, has led us to the conclusion that the effects of these waves, apart from slight heating, are purely mechanical. If intense enough, practically all cells can be cytolyzed. It is as if one could grasp a cell in both hands and bend it violently back and forth at a very rapid rate. Delicate structures on the outside of a cell are torn off. If the cell is very small it is thrown into nodes so quickly as to escape injury. If the cell can be held fixed and is not too viscous, its contents can often be made to whirl before it breaks down.

From the whirling one can gain an idea of the viscosity of the cell contents. Perhaps the chief value of the waves for biological investigation lies in the evidence obtained from their action regarding the viscosity of cells. It should be emphasized, however, that comparative studies of viscosity are difficult because of the great complexity of the sound wave patterns under the cover slip, both horizontally and vertically. Two cells in different portions of the same microscopic field are not necessarily exposed to the same radiational forces and great caution must be used in drawing conclusions regarding viscosity or resistance to tearing by difference in behavior of cells.

High frequency sound waves offer a new means of affecting the interior of cells without necessarily breaking down the cell wall. They will be of most value when a beam of given frequency and controlled intensity can be sent through a cell or tissue in a particular direction.

APPENDIX.

For those biologists who desire to construct a low-powered oscillator, the following constructional details ought to suffice.

The following apparatus is recommended.

One No. 852 Radiotron 75 watt tube,
One tube holder
One filament transformer to give 10 volts
One plate transformer to give 2,000 volts
One 5,000 ohm resistance
Several transmitting condensors (designed to withstand 5,000 volts) with an aggregate capacity of about 0.1 microfarad
One rheostat
Some heavy copper strip to wind the inductance
Some fine wire to make the secondary

All of the above can be bought from any radio store carrying parts for transmitting sets, and should not cost more than $100 in the aggregate.

Fig. 4 shows the wiring diagram and a suggested arrangement of the parts. The iron of the transformers should be on the side of the tube away from the oscillating parts and should be at least a foot from the tube. All the parts can conveniently be mounted on a board 30 x 10 inches.

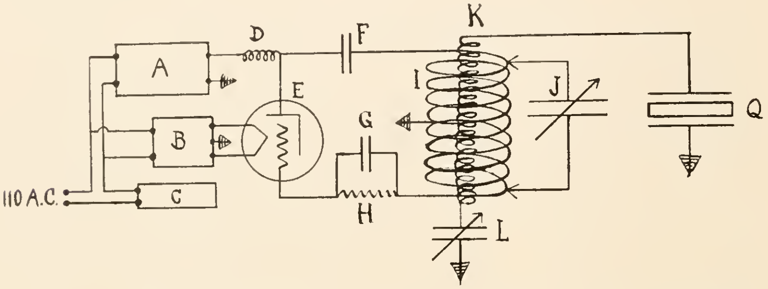


FIG. 4. Constructional diagram for a 75 watt oscillator. *A*, plate transformer; *B*, filament transformer; *C*, rheostat; *D*, choke coil; *E*, Radiotron No. 852; *F*, Blocking condenser. *G*, Grid leak condenser. *H*, Grid lead. *I*, Inductance coil; *J*, tuning condenser; *K*, secondary coil; *L*, Variable condenser; *Q*, Quartz plate between electrodes.

The rheostat should be mounted in the lead from the 110-volt A. C. house circuit and can be used to regulate the voltage. The primaries of the transformers should be connected in parallel across the house circuit. One side of the secondary of the plate transformer should be connected to the center tap of the filament transformer which point should also be grounded. The other side of the secondary should go through a choke coil to the plate. The choke coil can be made by winding about 100 turns of fine wire on a bakelite tube one or two inches in diameter.

The inductance can be made by winding fifteen or twenty turns of heavy copper wire on a bakelite tube six or eight inches in diameter. The plate should be connected to one tap on the inductance through a blocking condenser of about .002 microfarad capacity. The grid should be connected to the other tap on the inductance through a by-pass condenser of about the same capacity and a grid leak of above 5,000 ohms resistance. The center tap of the inductance should be grounded. The secondary can be made by winding 100 turns of fine wire on a bakelite tube which can be slipped inside the primary inductance. One end of the secondary should go to one plate of the crystal holder (the other plate of the holder being grounded). The other end of the secondary should be connected to ground through a variable condenser or to a rod of metal perhaps 1 inch diameter and ten inches long, which is not grounded.

The quartz crystal need not be larger than one square inch. It should be cut perpendicular to an electric axis. Its natural frequency of oscillation will depend on its thickness.

Mm. Thick.	Frequency (Approx.).
1	2,900,000 cycles per sec.
2	1,450,000 " " "
3	966,000 " " "
4	725,000 " " "
5	580,000 " " "
etc.	etc.

The oscillating circuit should be tuned to approximately the frequency of the crystal.

The crystal holder can conveniently be made out of two microscope slides and two thin brass strips with holes cut in them for use with the microscope. The microscope should be at least three feet from the oscillator so that movements of the operators body shall not change the frequency. The high tension lead to the microscope should be shielded by surrounding it with a grounded metal tube and the microscope itself should be grounded to prevent small spark discharges to the observer.

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[1] Kindly supplied by Dr. J. A. Dawson of Harvard University.

[2] Unpublished data of Dr. Otto Glaser of Amherst College.

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